Advances in Ex Vivo Modified Cell Therapies

1. Development of an Optimized Lentiviral Transduction Process for *Ex Vivo* CD34+ Hematopoietic Stem Cell Gene Therapy Drug Product Manufacture

Saranya Elavazhagan[1], Maria del Mar Masdeu[1], Tomasz Zabinski[1], Lily Du[1], Florence Enjalbert[1], Valentina Pennucci[1], Batika Rana[1], Christopher Whiting[1], Chiara Recchi[1], Paul Heal[1], Adrian J. Thrasher[2], Denise A. Carbonaro-Sarracino[1], Jia Wolfe[1], Bobby Gaspar[1], Pervinder Sagoo[1]

[1]Discovery Research, Orchard Therapeutics Europe Ltd, London, United Kingdom,[2]UCL GOS, Institute of Child Health, London, United Kingdom

Gene therapy using transplantation of autologous *ex vivo* gene modified $CD34^+$ hematopoietic stem cells (HSC) as an approach to treat a range of monogenic disorders, is now recognized for its transformative potential through several clinical studies and regulatory approvals. Despite significant advances in the commercialization of gene therapies, the main barriers to patient accessibility are the current limited capacity for manufacture of GMP-grade lentiviral (LV) vectors, and the corresponding substantial costs incurred by production of gene and cell therapies. This is further compounded by the need to manufacture higher numbers of gene modified HSCs for adult patients such as those suffering from X-Linked Chronic Granulomatous Disease (X-CGD), and for indications with larger patient cohorts. Reducing vector requirements and cost of goods therefore presents a key challenge in commercializing gene therapies. Application of transduction enhancers enables the use of lower quantities of LV vector to achieve the same output of gene modified cells. Several enhancer compounds are already routinely applied in clinical gene and cell therapy manufacture to improve the viral transduction process at various cellular levels, such as viral attachment, vector entry, and genome integration. To develop an optimized protocol for LV transduction of HSCs, we have screened over 20 commercially available and novel candidate compounds for enhancement activity, when applied individually or in combination to target distinct viral transduction pathways. Our comprehensive survey of improvements in transduction efficiency (TE) and vector copy number (VCN) achievable by these enhancers was conducted with both scale-down high-throughput and clinical-scale transduction processes for HSC gene therapy drug product manufacture, using clinical-grade therapeutic LV vectors. The most potent enhancer combinations were then assessed for compatibility with other known transduction culture process modifications, to develop an optimized protocol for HSC transduction. Enhancer treated HSCs were subject to extensive *in vitro* and *in vivo* characterization, including RNAseq transcriptional profiling and competitive engraftment studies in mice. Here we describe J-Boost, a representative compound from a novel class of transduction enhancers (diblock copolymers, PCT/US20/56123) which facilitates viral entry. When used in concert with Protamine Sulphate (PS) and high-density cultures, J-Boost results in up to ~9 fold increases in VCN and ~4 fold increases in TE, enabling 50-70% reduction in LV vector for HSC transduction to achieve desired target drug product profiles. Transduction is further enhanced by use of Retronectin™ coated culture vessels. Our results demonstrate that J-Boost/ PS enhancers are largely inert, inducing minimal alteration of HSC gene expression, HSC phenotype and multilineage progenitor function, while generating gene modified HSCs with comparable qualitative and quantitative HSC engraftment potential in NSG mice. Importantly, we show compatibility of this optimized transduction protocol with therapeutic LV vectors and manufacture methods currently in use for drug products under development for Beta-thalassemia, Mucopolysaccharidosis I (MPS I) and X-CGD. Demonstration of the safety, efficacy and comparability of this optimized transduction protocol validates its potential for clinical application and the achievable reduction in vector usage and manufacturing costs.

2. Non-Viral Integration of Large Cargo in Primary Human T Cells by CRISPR/Cas9 Guided Homology Mediated End Joining

Matthew J. Johnson, Beau R. Webber, Nicholas Slipek, Walker S. Lahr, Xiaohong Qiu, Blaine Rathmann, Miechaleen D. Diers, Bryce Wick, R Scott McIvor, Branden S. Moriarity

University of Minnesota, Minneapolis, MN

Adoptive cell therapy (ACT) using genetically engineered immune cells, such as CAR-T cells, holds tremendous promise for the treatment of advanced cancers. Current methods for the manufacturing of these cells rely on the use of viral vectors, greatly increasing manufacturing time, expense, and complexity. Furthermore, these viral vectors integrate in a non-site specific manner reducing functionality and raising safety concerns. Here we describe methods for efficient CRISPR-based, non-viral engineering of primary human T cells that overcome key limitations of previous approaches, namely DNA-induced toxicity and low efficiency integration of large genetic cargos. By synergizing temporal optimization of delivery, reagent composition, and integration mechanism, we achieve targeted knockin of cargo ranging from 1 to 3 kilobases at rates of up to 70% at AAVS1 (Figure 1), with post-editing cell viability of over 80%; efficiencies nearing those of viral vector platforms. Notably, approaches utilizing homology mediated end joining (HMEJ) and shorter homology arms (48bp) consistently outperformed those using longer 1kb homology arms and traditional homologous recombination. Off-target editing and integration were evaluated using GUIDE-seq and targeted locus amplification (TLA), respectively. As proof of concept, we engineered CAR-T cells and transgenic TCR T cells using a splice acceptor gene construct and gRNA specific to the TRAC locus, such that the CAR or transgenic TCR is expressed under the control of endogenous TRAC regulatory elements. Using this approach we consistently achieved integration

rates of over 20% for CAR-T cells and over 25% for TCR transgenic T cells (Figure 2). Additional optimizations, including culturing cells with anti-CD3 and anti-CD28 antibodies immediately following electroporation, further increased integration, averaging 39% for CAR templates. Furthermore, we demonstrate that these cells remain highly functional, retaining low expression of exhaustion markers, excellent proliferation and cytokine production capacity, and

potent anti-tumor cytotoxicity equal to or better than cells generated using a viral vector. Most importantly, these methods result in minimal-to-undetectable off-target editing and are readily adaptable to cGMP compliant and clinical-scale manufacturing. This non-viral gene engineering protocol offers a realistic, near-term alternative to the use of viral vectors in the production of genetically engineered T cells for cancer immunotherapy, offering immense potential for reducing manufacturing time, cost, and complexity compared to viral vectors without compromising cell expansion or function, while potentially increasing safety and efficacy via targeted integration.

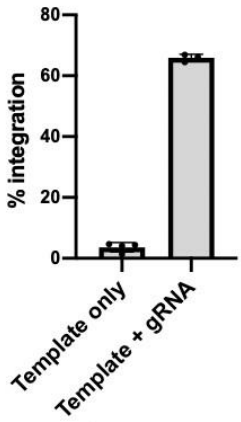

Figure 1. Expression of GFP in primary human T cells 8 days after transfection with Cas9 mRNA and a DNA minicircle template with 48bp of homology to AAVS1 encoding splice acceptor-GFP in the presence or absence of AAVS1 gRNA. GFP expression measured by flow cytometry.

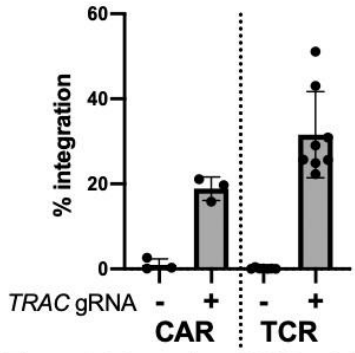

Figure 2. Expression of CAR or TCR in primary human T cells 7 days after transfection with Cas9 mRNA and a DNA minicircle template targeting TRAC and encoding CAR or TCR in the presence or absence of TRAC gRNA. Expression measured by flow cytometry.

3. Epigenetic Modulation of Aging to Increase CAR-T Cell Fitness

Benedetta Nicolis di Robilant, Rashmi Bankoti, Hazal Pektas, Lasse Jørgensen, Andreas Kongsgaard, Maddalena Adorno

Dorian Therapeutics, San Carlos, CA

Background: CAR-T cell therapy has had incredible clinical success in the treatment of hematological malignancies. However, very limited activity against solid tumors has been achieved so far, despite targeting a variety of antigens and tumor types. Here we show the impact of reducing cellular senescence in CAR-T cells to improve T cell fitness. Increasing evidence shows that immunosenescence is an important state of T cell dysfunction distinct from exhaustion; solid

tumors strongly induce senescence as a key strategy to evade immune surveillance and sustain a suppressive tumor microenvironment. Notably, the *ex vivo* manufacturing process of CAR-T cells also induces senescence extremely quickly; 15 days of T cell expansion age cells 30 years, as measured by telomere length, T cells differentiation and CDKN2a mRNA levels. Here we show that rejuvenating T cells by blocking cellular aging at the epigenetic level targeting USP16 is a powerful strategy to improve T cell fitness and clinical outcomes. Methods and Results: T cell aging is a very early phenomenon upon T cell activation, and it is drastically accelerated during ex vivo CAR-T manufacturing, resulting in a huge reduction of their ability to expand and kill cancer cells. In line with this hypothesis, we demonstrated that reducing cellular senescence increases CAR-T cell functions both in vitro and in vivo. USP16 is a deubiquitinating enzyme responsible for the removal of ubiquitin moieties from histone H2AK119, increasing chromatin accessibility to pro-senescent programs. Here, we show that targeting USP16 by means of a shRNA co-expressed within a CD19 or a GD2.CAR construct reduces T cell aging and increases stem cell memory (Tscm) frequency during manufacturing, without affecting proliferation. USP16 modulation also results in increased killing, polyfunctionality, and expansion upon in vitro stimulation with tumor cells. Notably, the delay of cellular senescence induces long-lasting cellular fitness as T cells are less exhausted upon multiple tumor challenges. Finally, T cells rejuvenated by USP16 modulation, show a strong increase in anti-tumor activity in an in vivo model of leukemia and neuroblastoma. **Conclusions:** Preventing T cell senescence by modulating the expression of USP16 increases self-renewal and antitumor activity, significantly improving the efficacy of CAR-T therapy. Development of small molecules against USP16 could offer a viable solution to improve T cell fitness during manufacturing.

4. Effective and Efficient Intracellular Delivery Achieved with the Cell Squeeze® Technology Enables Rapid, Scaled, and Reproducible Production of Cell Therapies

David Chirgwin, Maisam Dadgar, Jason Murray, Claire Page, Scott Loughhead, Ipsita Roymoulik, Howard Bernstein

SQZ Biotechnologies, Watertown, MA

Personalized cellular therapies, especially in cancer, have grown in number over the last five years. Cell therapies require precise biological engineering using cargos which are intracellularly delivered. However, conventional approaches for delivering materials into cells are limited in the types of biology they can engineer and have manufacturing challenges with regards to scale and reproducibility. The Cell Squeeze® technology uses microfluidic chips with constrictions to deform cells resulting in the effective intracellular delivery of molecules to cells while preserving cell health and function. This technique enables numerous opportunities to reproducibly generate new classes of cell therapies at scale. We have implemented the Cell Squeeze® process for cGMP and aseptic manufacturing. Our system is appropriate for use in a Grade B cleanroom utilizing a single-use sterile disposable kit containing multiple microfluidic chips. The Cell Squeeze® manufacturing system can accommodate up to 20 billion cells for processing in 2 minutes or less. Cell viability at manufacturing scale is >90% and the percent of the cell population delivered is >80%. This system is currently being used to manufacture drug product in a Phase 1 clinical trial using peripheral blood mononuclear cells (PBMCs) to generate antigen presenting cells as a cellular vaccine. To further enhance accessibility of SQZ Biotechnology cell therapies, we are also developing a point-of-care manufacturing system with the potential to manufacture cell therapies for same-day dosing. Our advances in cell therapy manufacturing will potentially enable multiple therapeutics across oncology, infectious disease, and immune disorders to be developed and delivered to a broad patient population.

5. Sequential CRISPR-Mediated Engineering and Clonal Banking for the Generation of Multiplexed Engineered Master Pluripotent Cell Lines for the Mass Manufacture of Offthe-Shelf Immune Cells Targeting Solid Cancers

Ramzey Abujarour, Lauren K. Fong, Fernanda R. Cugola, Nicholas Brookhouser, Chelsea M. Ruller, Berhan Mandefro, Janel Huffman, Karma Farhat, Tom T. Lee, Bahram Valamehr

Fate Therapeutics, San Diego, CA

Chimeric antigen receptor (CAR) T and NK cell therapies have shown great promise in treating blood malignancies, but tackling solid tumors is hindered by a shortage of targetable tumor-specific antigens, antigen escape, and poor activity of expanded patient-derived immune cells. While a multiplexed engineering approach can arm immune cells to address these obstacles, engineering patient- or donor-derived NK and T cells often leads to inconsistent, heterogeneous, and costly products. We describe here a platform to enable complex genetic engineering and precise targeting of independent loci through sequential rounds of CRISPR-mediated editing, single cell cloning, screening and banking of induced pluripotent stem cells (iPSCs) leading to the generation of multiplexed engineered master iPSC clones. Using this platform, we are developing a master iPSC bank that is uniformly engineered with four anti-tumor modalities. A core of three edits are first introduced and include: i) IL-15 receptor fusion protein (IL-15RF) for enhanced NK cell activity, ii) high affinity non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity, and iii) CD38 deletion for enhanced NK cell function. The fourth edit, CAR targeting the HLA-I related molecules MICA and MICB (MICA/B), is engineered

next in a second locus for pan-tumor targeting. Challenges with building such a complex cellular therapy include genomic instability and off-target editing that could be introduced during CRISPR/nuclease-based engineering of multiple loci, and during the multiple

rounds of single cell cloning, expansion, and banking. To reduce offtarget edits that could be caused by simultaneously targeting two loci, we performed sequential engineering instead, targeting one locus at a time to build a more complex master iPSC line. In the first step of the engineering process, iPSCs were engineered with the three core antitumor modalities by targeting an IL-15RF-hnCD16 cassette into the CD38 locus, resulting in complete CD38 gene disruption. The CD38 gene was targeted with fully characterized gRNA and donor plasmid containing the IL15-RF-hnCD16 cassette. Engineered iPSCs were cloned from single cells and clones were screened and fully tested before and after banking. Out of 257 iPSC clones screened, 88 clones (34%) had the IL-15RF-hnCD16 transgenic cassette targeted specifically into the CD38 gene. Evaluation of genomic stability of the selected clones after banking revealed that 12 of the 15 selected clones maintained genomic stability as determined by G-banded karyotyping, and 10 clones out of 11 tested showed no genomic copy number variations as determined by a genome-wide SNP microarray analysis. Banked iPSC clones were further evaluated for pluripotency and propensity to differentiate into NK cells which were further tested for phenotype and function. The best performing clone was then selected for a second round of genetic engineering, whereas CAR-MICA/B is inserted into a safe harbor locus at the single cell level to create the final master iPSC clone containing the three core edits and the CAR-MICA/B. The generated engineered iPSC subclones are currently being screened for specific targeting of the CAR-MICA/B into the safe harbor locus and other critical quality attributes (including maintenance of pluripotency and genomic stability) to nominate the final iPSC master cell bank. This master cell bank will serve as the starting material for clinical investigation of FT536, an off-the-shelf pan-cancer targeting CAR NK cell immunotherapy with potential therapeutic application to multiple hematopoietic and solid tumors.

6. Generation of Engineered Tregs (EngTregs) from Umbilical Cord Blood Derived CD4⁺ T Cells via HDR-Dependent *FOXP3* Gene Editing

Shivani Patel[1], Yuchi Honaker[1], Su Jung Yang[1], Noelle P. Dahl[1], Warren Anderson[1], Samuel Scharffenberger[1], Iram Khan[1], Michelle Christian[1], Karen Sommer[1], David J. Rawlings[1,2,3]

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cell doses and maintaining cell purity and Treg stability. We previously developed gene engineered Tregs (EngTregs) from PB-derived CD4⁺ T cells as an alternative source for Treg cell therapies. This approach utilizes co-delivery of a *FOXP3*-targeting designer nuclease and an rAAV homology-directed-repair (HDR) donor template designed to introduce a gene cassette containing MND promoter and cis-linked LNGFRt surface tag into the *FOXP3* locus. The resulting enforced and stable expression of FOXP3 reprograms HDR-edited cells to acquire a Treg-like phenotype and suppressive function in vitro and in vivo in xeno-GvHD mouse models. This approach permits clinical-scale production of highly purified EngTregs for potential therapeutic use. The naïve phenotype of CD4⁺ T cells in UCB suggests a potential proliferation and potency advantage over T cells in adult PB as a source for EngTregs production. Further, HDR editing in UCB T cells might provide the capacity to generate multiple cell doses for shortterm allogeneic cell therapies. In the current study, we evaluated the feasibility of using UCB-derived CD4⁺ T cells to generate EngTregs. We found that UCB-derived CD4⁺ T cells could be edited at a high efficiency. Edited cells could also be efficiently enriched by the surface LNGFRt selection marker. Notably, the expansion methods optimized for PB-derived EngTregs (rapamycin treatment, high dose of IL-2 and G-rex culture) did not yield comparable cell viability or quantity for UCB-derived EngTregs. Therefore, we established alterative protocols for cell expansion; approaches that significantly improved the yield and viability of the UCB EngTregs products. In parallel with these studies, we also tested an alternative editing strategy designed to introduce a heterodimeric, chemically-induced signaling complex (CISC; that mimics IL-2 signaling in response to an exogenous dimerizer) upstream of the *FOXP3* gene. UCB derived EngTregs cells products (including LNGFRt+ or CISC+ EngTregs, respectively) exhibited high purity, stable FOXP3 expression, consistent expression of key Treg markers and limited expression of proinflammatory cytokines upon stimulation. Additionally, we tested whether UCB-derived EngTregs could suppress allogeneic effector T cells in the xeno GvHD mouse model. Our preliminary data showed that the UCB-derived EngTregs have suppressive capabilities against HLA-mismatched allogeneic effector T cells in NSG recipient mice. In summary, our data demonstrate a robust capacity to engineer UCB-derived T cells leading to generation of EngTregs that exhibit a stable Treg phenotype and function. Additional optimization of cell expansion is likely to provide cell yields that enable UCB to be a viable future cell source for EngTregs.

7. VOR33: A Clinic-Ready CRISPR/Cas9 Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia

John Lydeard[1], Michelle Lin[1], Chong Luo[1], Shu Wang[1],

[1] Center for Immunity and Immunotherapies and Program for Cell and Gene Therapy, Seattle Children's Research Institute, Seattle, WA,[2]Pediatrics, University of Washington School of Medicine, Seattle, WA,[3]Immunology, University of Washington School of Medicine, Seattle, WA

Regulatory T cells (Tregs) play a crucial role in peripheral immune tolerance and homeostasis. Adoptive transfer of Tregs is being pursued in clinical trials for autoimmune diseases and for bone marrow and solid organ transplantation. Current Treg trials utilize ex-vivo expanded thymic Tregs (tTregs) derived from either peripheral blood (PB) or umbilical cord blood (UCB); however, this approach must overcome technical challenges that include manufacturing adequate

Amanda Halfond[1], Mark B. Jones[1], Julian Scherer[1], Dane Hazelbaker[1], Meltem Isik[1], Azita Ghdossi[1], Juliana Xavier-Ferrucio[1], Gary Ge[1], Elizabeth Paik[1], Gabriela Zarraga-Granados[1], Taylor Perkins[1], Matthew Li[1], Brent Morse[1], Siddhartha Mukherjee[2], Sadik Kassim[1], Tirtha Chakraborty[1]

[1]Vor Biopharma, Cambridge, MA,[2]Columbia University Medical Center, Columbia University, New York, NY

Introduction: AML is the most common form of adult acute leukemia, with median 5-year survival rate <30%. Allogeneic hematopoietic cell transplant (HCT) has long been the standard of care for high-risk patients (pts), with >3500 transplants performed annually in the US. There is unmet need for new treatments in ~40% of pts who relapse. With existing targeted therapies, cell surface marker expression between cancer and normal cells is not differentiated enough to limit "on-target, off-tumor" toxicity. Antigens (Ag) (eg, CD33) expressed on normal myeloid cells and/or progenitors (Levine et al. 2015) confer dose-limiting toxicity of Ag-directed therapies in AML. To unlock the full potential of targeted treatments, we create treatmentresistant hematopoietic stem cells (HSCs) by genetically ablating CD33 from healthy, HLA-matched (10/10) donor HSCs followed by HCT, creating a target Ag-negative hematopoietic system. The reconstituted hematopoietic compartment of pts receiving CD33-null cells will be resistant to cytotoxicity induced by Mylotarg™, an antiCD33 monoclonal antibody conjugated with cytotoxic calicheamicin. Human CD33 null hematopoietic cells show no impairment of function and are resistant to CD33-targeted therapies (Borot et al. 2019; Humbert et al. 2019; Kim et al. 2018). Notably, this is consistent with natural genetic evidence of CD33 null humans with no deleterious phenotype (gnomad.broadinstitute.org/). Here, we describe the preclinical data and process scale-up of the CD33-null HSC graft (VOR33) for a first-in-human clinical trial. **Methods/Results:** The manufacturing process yielded clinically relevant doses of VOR33 (>3x10^6 viable CD34+ cells/kg) under GMP-like conditions with GMP-appropriate reagents. CD34+ cells, isolated from G-CSF and plerixafor mobilized peripheral blood leukapheresis products, were edited using CRISPR/Cas9 to disrupt *CD33* gene. At scale, we routinely achieved gene knockout of >70% (90% biallelic) with no loss of cell viability. Cells differentiated from VOR33 displayed normal myeloid markers, phagocytosis potential and induction of inflammatory cytokines equivalent to unedited (CD33+) control cells. Phenotypic and functional characterization revealed no difference in frequency of long-term HSCs in VOR33 vs unedited controls. Pharmacology studies using NOD/SCID-gamma mice, with VOR33 cells manufactured under GMP-like conditions, showed normal long-term engraftment (16-week bone marrow chimerism of 83.1±9.0% vs 87.9±7.3% in control group) and multilineage differentiation. In addition, we observed persistence of VOR33 gene editing and preservation of indel species distribution after 16 weeks, indicating no counterselection or clonal expansion of CD33-null cells. Importantly, we found loss of CD33 protein conferred selective protection to VOR33-derived myeloid cells vs Mylotarg *in vitro* (>65-fold) and *in vivo* (>60-fold). In our GLP toxicology study of >40 tissues, we saw no tumorigenicity or notable changes in toxicology parameters. In-depth genotoxicity analyses were carried out with a subset of scaled-up manufactured lots of VOR33, including those used for toxicology and pharmacology studies. Deep sequencing of 2369 genomic sites, by homology-dependent and independent methods, revealed no off-target editing. **Conclusion:** These studies set the stage for initiation of, as well as evaluation of safety and efficacy in, a multicenter first-in-human clinical trial of VOR33 in pts with AML.

Cancer - Oncolytic Viruses

8. Preclinical Toxicology Assessment of an Oncolytic Measles Virus Armed with *H. pylori* Immunostimulatory Bacterial Antigen in Preparation for a Phase I Trial in Breast Cancer Patients

Kimberly Viker[1], Michael B. Steele[1], Ianko D. Iankov[1],

[1] Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[2]GEMpath, Inc., Longmont, CO,[3]Division of Medical Oncology, Mayo Clinic, Rochester, MN

Introduction: Despite recent therapeutic advances, metastatic breast cancer (MBC) remains incurable. Engineered measles virus (MV) strains based on the attenuated MV Edmonston vaccine platform have demonstrated significant oncolytic activity against solid tumors. *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is responsible for the robust inflammatory reaction in the gastroduodenal mucosa during infection. NAP attracts and activates immune cells at the site of infection inducing expression of inflammatory mediators. Our team engineered an MV strain expressing the secretory form of NAP (MV-sNAP) that exhibits anti-tumor and immunostimulatory activity in human breast cancer xenograft models. In this study, we investigated the biodistribution and toxicity of MV-sNAP in MV-susceptible transgenic Ifnarko™-CD46Ge mice. The primary objectives were to identify potential toxic side effects and to define the optimal equivalent dosage of MV-sNAP prior to proceeding with a Phase I clinical trial in MBC patients. **Methods:** Ninety-six, 5-to 6-week-old female Ifnarko™CD46Ge mice were stratified into 6 groups with 2 cohorts (48 mice/ cohort). Treatment with MV-sNAP (doses: 10^6 or 10^7 $TCID_{50}$/mL) or vehicle control was started on day 0 using either a single subcutaneous (SC) or intravenous (IV) injection (day 0) or via 3 repeated SC or IV injections on days 0, 14, 28. Body weight and clinical signs of toxicity were monitored daily, and hematology, plasma chemistry, plasma cytokines, gross pathology, and histopathology of major organs were analyzed on days 11, 12, 54 or 56 of the study. The immune response to MV-sNAP was assessed by a NAP-mediated ELISA virus neutralization test. Biodistribution of MV-sNAP was evaluated by qRT-PCR. **Results:** All mice survived to their respective endpoints, with no evidence of

Arun Ammayappan[1], Susanna C. Concilio[1], Brad Bolon[2], Nathan J. Jenks[1], Eleni Panagioti[1], Mark J. Federspiel[1], Minetta C. Liu[3], Kah Whye Peng[1], Evanthia Galanis[3]

clinical toxicity. No significant difference between control and treated animals was observed in complete blood counts or plasma chemistry values including liver function at all time points. Low MV genome copy numbers of MV-sNAP were found on Day 11 in the inguinal lymph nodes (ILN) in 2 of 8 animals that received a single SC low dose injection, and in 3 of 8 animals that received a single SC high dose injection. In contrast, a single IV administration of MV-sNAP resulted in significant MV genome copy numbers in most tissues on day 11 in both dose groups. In animals given three SC MV-sNAP injections, the low-dose group exhibited expression only in the spleen and ILN in 1 of 8 animals on day 56. By day 12, a single IV administration of MV-sNAP resulted in a strong early immune response to MV antigens in all mice of both dose groups. High anti-MV titers were maintained by day 54 following three IV injections in all animals of both dose groups. MV-sNAP did not significantly increase circulating levels of pro-inflammatory cytokines. Histopathologic findings following administration of MV-sNAP showed asymptomatic minimal or mild hemorrhage in the lung following SC or IV administration (1-2/8 animals on day 11), and minimal leukocyte infiltration/inflammation at the SC injection site. These modest changes were interpreted to be non-adverse. **Conclusion:** Both SC and IV delivery of MV-sNAP were well tolerated, and no significant toxicity was observed in a relevant (i.e., MV-susceptible) engineered mouse model. This outcome supports the safety of the MV-sNAP platform for oncolytic virotherapy of MBC. Enrollment in a Phase I clinical trial of MV-sNAP in patients with MBC (NCT 04521764) started in November 2020.

9. Validating Secreted IFNβ as an In Vivo Biomarker of Intratumoral Replication of VSV-IFNβ-NIS

Lianwen Zhang, Michael B. Steele, Nathan J. Jenks, Alysha N. Newsom, Martha Q. Lacy, Kah Whye Peng, Stephen J. Russell

Mayo Clinic, Rochester, MN

VSV-IFNβ-NIS is an oncoytic vesicular stomatitis virus which is currently being evaluated in multiple clinical trials. Infection of target tissues can be monitored using SPECT or PET imaging to detect expression of the NIS transgene. IFNβ was encoded in the virus to limit its replication in normal tissues and to enhance the inflammatory/ immune response in the infected tumor. However, we demonstrate here in murine models that IFNβ can also serve as a convenient soluble biomarker of VSV-IFNβ-NIS replication in vivo. We first evaluated the baseline level of IFNβ that is produced by innate immune cells upon intravenous administration of VSV. Infusion of 1e8 $TCID_{50}$ VSVhIFNβ-NIS in tumor free C57BL mice induced a transient increase of endogenously secreted mIFNβ, between 32-64 pg/ml at 24h, and is undetectable at 48h. In contrast, the level of hIFNβ produced by VSV-hIFNβ-NIS infected cells in tumor free mice was 500-2000 pg/ ml at 24h but is significantly higher in 5TGM1 tumor bearing animals (1000-65,000 pg/ml). The difference in tumor bearing versus tumor free mice becomes more significant at 48h where the virus continues to replicate and spread in the permissive 5TGM1 tumors. Varying doses of VSV-mIFNβ-NIS was also given intravenously to mice bearing 5TGM1 or MPC11 tumors, and blood levels of IFNβ were measured. Results indicate that the level of virally encoded IFNβ in tumor bearing mice is

dose dependent. There was a corresponding increase in median mIFNβ detected in the plasma of 5TGM1 mice given 1e8, 3e8 and 1e9 $TCID_{50}$ VSV-mIFNβ-NIS. Importantly, the level of IFNβ remained high over days 1-3, and started to decline from day 4, suggesting that secreted IFNβ could be a valuable biomarker to monitor the kinetics of viral infection in cells, followed by subsequent extinction of the infection and death of infected cells. We did not observe a secondary wave of infection in these immunocompetent animals. By day 7, there was no detectable levels of mIFNβ. Corresponding anti-VSV antibody titers are detectable by day 7, and plateaued by day 15. Interestingly, when we compared intratumoral versus intravenous administration of 3e8 $TCID_{50}$ of virus, we found overall higher levels of plasma mIFNβ in the intravenous group versus the intratumoral group, suggesting that systemic delivery of an oncolytic virus might be more beneficial to allow a more uniform seeding of the tumor parenchyma and infectious foci. In the MPC-11 plasmacytoma model in Balb/c mice, we saw a significantly higher level of plasma IFNβ, indicating a much more permissive tumor substrate for viral replication, and/or rapid death and release of cellular contents. In summary, our study confirms that virally encoded IFNβ produced by VSV-IFNβ-NIS infected cells can serve as a convenient and simple biomarker of viral replication in vivo, and that the level and profile (duration) of IFNβ in the plasma could serve as an early indicator of the relative permissiveness of the tumor substrate to the oncolytic virotherapy. These findings in the murine models are recently corroborated by early findings in the intravenous VSV-IFNβ-NIS Phase I trials in cancer patients.

10. Development of Novel Oncolytic Vector Based on Alternative Adenovirus Serotype 6 for Glioblastoma and Breast Cancer Therapy

Margarita Romanenko[1,2], Ivan Osipov[2], Sergei Kutseikin[2], Mariia Sizova[2], Anastasiya Paramonik[2], Antonina Grazhdantseva[3], Galina Kochneva[3], Julia Davydova[1], Sergey Netesov[2]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]Novosibirsk State University, Novosibirsk, Russian Federation,[3]State Research Center of Virology and Biotechnology "Vector", Koltsovo, Russian Federation

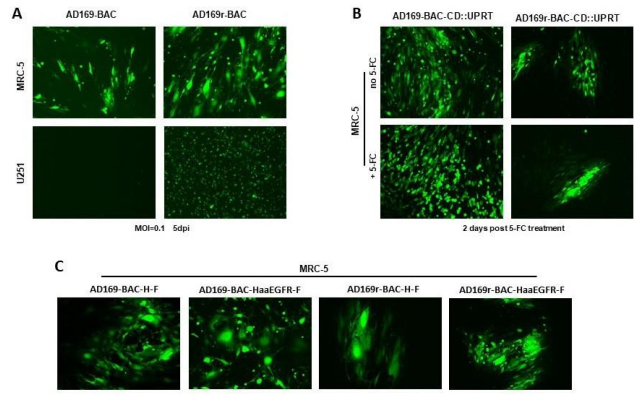
The majority of adenovirus oncolytic agents are based on the adenovirus serotype 5 (Ad5), which is the most studied and the most used type of Ad in the field of gene therapy. However, there is a

constant search for alternative Ad types, which are able to overcome the existing obstacles hampering the Ad5 application such as high seroprevalence and rapid clearance of viral particles after i.v. administration. Among many examined alternative Ad serotypes, adenovirus serotype 6 (Ad6) exhibits high oncolytic efficacy against many cancer types. Importantly, Ad6 was shown to have a lower seroprevalence and improved ability to escape from Kupffer cells when compared to Ad5. We recently demonstrated that wild-type Ad6 exhibits a dose-dependent cytotoxicity against glioblastoma cells *in vitro* and a significantly improved therapeutic effect in U87MG xenografts after intratumoral injection, which was comparable of that with Ad5 wild-type control vector. To further enhance the therapeutic efficacy of Ad6, we constructed a tumor-specific recombinant vector Ad6hTERT-GMSCF with the E1 region controlled by the human telomerase reverse transcriptase promoter (hTERT) and the human granulocytemacrophage colony-stimulating factor (GMCSF) transgene expressed from the Ad E3 region. The immunomodulatory activity of secreted GMCSF was evaluated after infection A549 lung adenocarcinoma cells with Ad6-hTERT-GMSCF by assessing the stimulation level of human erythroleukemia cells TF-1 proliferation. The oncolytic efficacy of Ad6-hTERT-GMSCF *in vitro* and *in vivo* was evaluated on both triplenegative breast cancer (MDA-MB-231) and glioblastoma multiforme (U87MG) cell lines. The insertion of hTERT promoter and GMSCF into the Ad6 genome did not significantly affect the oncolytic potential of Ad6 vector as it was demonstrated by the cell viability test. The *in vivo* efficacy of Ad6-hTERT-GMSCF in comparison to wild-type Ad6 and Ad5 vectors was evaluated using subcutaneous MDA-MB-231 or U87MG xenografts in SCID mice after performing injections three times (day 1, 3, and 5). The new recombinant Ad6-hTERT-GMSCF was able to significantly inhibit the tumor growth in both MDA-MB-231 and U87MG models. Importantly, the therapeutic effect of Ad6-hTERTGMSCF was comparable with that of Ad5 and Ad6 wild-type control viruses. We are currently investigating the level of GMCSF in tumor xenografts. The oncolytic potential of a novel Ad6-hTERT-GMCSF vector needs further investigation in immunocompetent models. Further, additional genetic modification (e.g., fiber knob switching) may be beneficial for further enhancement of anticancer efficacy of Ad6-based recombinant vectors.

11. Human Cytomegalovirus Engineered for Glioma Therapy

Haifei Jiang, Rebecca Nace, Stephen Russell
Molecular Medicine, Mayo Clinic, Rochester, MN

Human cytomegalovirus (HCMV) is a 230nm diameter betaherpesvirus with a ~235kb double-stranded linear DNA genome, an icosahedrally ordered nucleocapsid, a tegument layer and a complex envelope. HCMV naturally hijacks neutrophils and monocyte/ macrophages for local spread, then infects endothelial cells and disseminates systemically in various hematological cell types. In the brain, HCMV infects glial cells and spares neurons. We are therefore interested to develop HCMV as an oncolytic platform for the treatment of glioma since it should infect both the tumor cells and tumor infiltrating macrophages and, if appropriately armed, may efficiently recruit cytotoxic T cells and NK from circulation to target CMV antigen positive cells within the glioma.To develop HCMV as a glioma-targeting vector we worked with the lab adapted strain AD169 and constructed a bacterial artificial chromosome (BAC) clone (AD169-BAC). Genome sequencing confirmed the expected sequence deletions between UL1-UL20 and of the UL/b´ region which in wtCMV encode protein and RNA products that combat T cell and NK cell activation promote virus latency in hematological cells. Vaccines based on lab adapted AD169 and Towne strains have good safety profiles in clinical trials. To enhance glioma cell targeting, we repaired the function of the gH/gL/UL128-131 glycoprotein complex by replacing the UL131 like sequence in the AD169 backbone with the UL131 ORF from Merlin strain (AD169r-BAC). Comparing AD169BAC and AD169r-BAC, both efficiently infected and killed MRC-5 cells, but only AD169r-BAC spread in U251 glioma cell cultures (Fig A). Both viruses showed antitumor activity in U87 and U251 glioma xenograft models, although AD169r-BAC was more potent. We next knocked out the US1-US11 region which encodes proteins and RNAs that inhibit MHC-I and MHC-II pathways and in vivo testing in U87 model suggested superior infectivity of these viruses. We next inserted the suicide gene CD::UPRT (cytosine deaminase fused to uracil phosphoribosyl transferase) into AD169-BAC and AD169rBAC (Fig B), and in parallel inserted untargeted and EGFR-targeted measles F/H glycoprotein complexes into the same backbones (Fig C). We are currently testing the efficacy and mechanisms behind these recombinant AD169 viruses expressing suicide genes or fusogenic F/H complexes in subcutaneous and orthotopic glioma models. Our preliminary data suggest that HCMV could be developed as a unique anti-cancer oncolytic or gene therapy platform, and more therapeutic transgenes will be added.



12. Virulent Velogenic Newcastle Disease Virus Is More Oncolytic Than Attenuated and Lentogenic Newcastle Viruses

Ahmed Majeed Al-Shammari[1], Sarah Hassan[2], Aida Bara Allawe[3]
[1]Experimental Therapy Department, Mustansiriyah University, Iraqi Center for Cancer and Medical Genetic Research, Baghdad, Iraq,[2]Baghdad University,
College of Veterinary Medicine, Baghdad, Iraq,[3]Department of Microbiology, College of Veterinary Medicine, Baghdad University, Baghdad, Iraq

Lentogenic LaSota strain, attenuated AMHA1 strain and AMHA2 virulent Iraqi strain of Newcastle disease virus (NDV) are three

replication-competent, non-recombinant natural oncolytic Newcastle disease viruses evaluated in the current study for their anticancer effects against breast cancer cells. All these strains selectively replicate within tumor cells. AMHA1 is attenuated NDV virus after 7 years of passaging in embryonated chicken eggs, carrying avirulent lentogenic fusion protein motif. Iraqi virulent strain AMHA2 carry virulent F protein cleavage site motif. In vitro cytotoxicity assay, clonogenic assay, replication curve, quantitative real-time PCR assay for NDV mRNA and cytopathic effect studies revealed that virulent AMHA1 strain was more effectively infected, replicated, and killed human and mouse breast cancer cells, followed by attenuated AMHA2 and lastly lentogenic LaSota being less effective. The virulent AMHA2 strains were able to induce more powerful apoptotic response as studied by acridine orange-propidium iodide apoptosis assay in breast cancer cells followed by attenuated AMHA1, in contrast to LaSota NDV strain which produce less apoptotic effect yet still significant. Immunohistochemistry performed on human and mouse breast cancer

cells revealed high NDV HN viral protein expression in cancer cells. To study the apoptosis mechanism induced by each strain, caspase-8 and caspase-9 protein expression were evaluated and found that caspase-9 showing higher expression induced by all three strains with association of caspase-8 which have lower expression. The use of more virulent strains in oncolytic virotherapy can be more efficient strategy for effective tumor treatment.

13. Generation and Characterization of Replication-Competent Oncolytic Foamy Virus Vectors

Karol Budzik, Rebecca Nace, Yasuhiro Ikeda, Stephen Russell

Molecular Medicine, Mayo Clinic, Rochester, MN

Simian Foamy Viruses (SFVs) are ancient, non-pathogenic retroviruses that infect a variety of nonhuman primates with sporadic cases of zoonotic transmission to humans. Similar to Gammaretroviruses replication of SFVs is limited to dividing cells, however, unlike Gammaretroviruses, SFVs have a remarkable ability to persist unintegrated at the centrosome of quiescent cells for weeks, waiting for nuclear membrane disintegration during cell division. This feature enables SFV to efficiently infect cells with long doubling times, such as human tumor cells, which have been reported to have doubling times between 25 to over 200 days. SFV's unique features make it a promising candidate for a useful human cancer therapy. Here, we describe the generation and characterization of oncolytic Simian Foamy Virus (oFV) vectors. oFV was generated by combining genome segments from 2 strains of the chimpanzee SFV - PAN1 and PAN2. oFV was then engineered to carry a reporter gene - GFP in place of the *bel-2* gene (oFV-GFP). Both the parental and the GFP-carrying virus replicated efficiently, albeit slowly, in a panel of human cancer cell lines *in vitro* and exhibited a clear oncolytic activity *in vivo.* A single dose of 10^7 IU of either vector potently controlled growth of orthotopic intraperitoneal ovarian cancer metastases and significantly prolonged survival of the treated mice. Subsequently, we armed oFV with a suicide gene, HSV1 Thymidine Kinase (TK), and tested *in vivo* in subcutaneous xenograft glioblastoma tumors engineered to express firefly luciferase in response to FV infection. Upon infection with oFV-TK and treatment with Ganciclovir, luminescence in the infected indicator tumors decreased, indicating accelerated death of the oFV infected cells. Our data show that oFV is a promising gene delivery platform and candidate for a cancer therapeutic.

14. An Oncolytic Adenoviral Vector Expressing an Anti-PD-L1 scFv Reduces Tumor Growth in a Melanoma Mouse Model

Maria Vitale[1,2], Eleonora Leggiero[2], Margherita Passariello[2], Anna D'Agostino[2], Lorella Tripodi[2,3], Claudia De Lorenzo[1,2], Vincenzo Cerullo[1,4], Lucio Pastore[1,2]

[1]Molecular Biology and Medical Biotecnology, University of Naples, Federico II, Napoli, Italy,[2]CEINGE, Naples, Italy,[3]European school of Molecular Medicine (SEMM), Naples, Italy,[4]3Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Oncolytic virotherapy is an emerging therapeutic approach, based on replication-competent viruses, to selectively infect and destroy cancer cells, causing the release of tumor-associated antigens (TAA), therefore stimulating an antitumoral immune response. Indeed, oncolytic adenoviruses (Onc.Ads) can kill cancer cells in different ways, primarily by inducing immunogenic cell death. To increase the anticancer activity of Onc.Ads, it is possible to combine them with a strategy aimed at blocking tumor immune evasion. Programmed death ligand 1 (PD-L1) is mainly expressed on tumor cells surface; binding to its receptor, PD-1, expressed on CD8+ T cell inhibits their proliferation and antitumoral activity. As demonstrated with monoclonal antibodies, a PD-L1 antagonist can prevent the immune escape T cell-mediated of the tumor cells. We developed an Onc.Ad expressing a single-chain variable antibody fragment (scFV) against PD-L1 to combine blockage of PD-1/PD-L1 interaction with the antitumoral activity of Onc.Ads. We confirmed the expression and secretion of scFv anti-PD-L1 in the supernatant of infected cells by western blot analysis. B16-OVA cells (a mouse melanoma cell model) were then infected with Onc.Ad5Δ24scFV-PD-L1 and treated with C57BL/6 splenocytes. We observed that treatment combination was significantly more effective in reducing cell viability. We then evaluated tumor progression *in vivo* in three groups of mice, inoculated with syngeneic B16-OVA melanoma cells, treated with either Onc.Ad5Δ24-scFV-PD-L1, Onc.Ad5Δ24 or mock treatment. We observed that treatment with Onc.Ad5Δ24-scFV-PD-L1was more effective in reducing melanoma growth compared to mice treated with Onc.Ad5Δ24. We will further immunologically characterize immune response induced by the combined treatment; however, these results suggest that Onc.Ad-induced expression of an immune checkpoint inhibitor is an effective and promising strategy.

Delivery Technologies and CRISPR for Therapeutics

15. CRISPR/Cas9-Mediated Targeted Gene Insertion Platform Achieves Durable, Normal Human Alpha-1 Antitrypsin Protein Levels in Non-Human Primates

Sean Burns, Jenny Xie, Adam Amaral, Darleny Lizardo,
Carri Boiselle, Catherine Moroski-Erkul, Kathryn Walsh, Tenzin Yangdon, Elena Kollarova, Vinita Doshi,
Riley Cole, Nikunja Kolluri, Shreelekha Jaligama, Kenneth Manning, Harini Sampath, Dohyun Kim, Palak Sharma, Trisha Das, Samantha Soukamneuth, Sucharitha Parthasarathy, Andrew Whynot, Richard Duncan, Lucinda Shaw, Matthew Roy, Michelle Young,
Noah Gardner, Yuanxin Xu, Laura Sepp-Lorenzino, Jessica Seitzer, Anthony Forget
Intellia Therapeutics, Cambridge, MA

CRISPR/Cas9-based genome editing offers the potential to address genetic diseases at their source. Using this technology, we have created a liver-directed, modular genome editing platform to enable rapid therapeutic advancement for genetic diseases with high unmet need, by either reducing a disease-causing protein through knockout editing or enabling production of a functional protein through targeted gene insertion. We have pioneered the use of lipid nanoparticles (LNPs) to enable systemic, transient, and well-tolerated delivery of the CRISPR/ Cas9 genome editing system. Using a hybrid approach combining LNPencapsulated CRISPR/Cas9 components with an adeno-associated virus (AAV) for "promoterless" donor DNA template delivery, we achieve targeted gene insertion resulting in high levels of protein expression *in vivo* in both murine and non-human primate (NHP) models. Here, we illustrate the power of our platform for advancing genome editing to treat alpha-1 antitrypsin deficiency (AATD). In this disease, mutations in the *SERPINA1* gene lead to liver pathology due to aggregation of the alpha-1 antitrypsin (AAT) protein in hepatocytes, and lung pathology due to deficiency of the AAT protein in the lungs. Normal levels of AAT protein in humans are approximately 1,0002,700 ug/mL, a high level that has been challenging to achieve using chronic protein augmentation therapy or traditional gene therapy approaches. By leveraging CRISPR/Cas9 to perform precise gene insertion downstream of the strong endogenous albumin promoter, we now demonstrate the sustained production of normal human levels of AAT protein in NHP, without impacting normal albumin levels. The AAT protein concentration rose quickly after a single administration, reaching the normal range by week 4, and remained stable within the normal range through week 14 in an ongoing study. The physiological levels of human AAT protein produced in this study are expected to be fully therapeutic to restore protease inhibition and protect the lungs in AATD. These results in NHPs build on our previous findings in a humanized mouse model of AATD in which we showed successful consecutive *in vivo* genome editing (*SERPINA1* knockout plus insertion) as an alternative approach to addressing both liver and lung manifestations of the disease. Moreover, they highlight the potential of our modular targeted gene insertion platform to produce durable, high-level expression of therapeutic proteins for diverse genetic diseases, beyond what has been achieved using traditional gene therapy approaches.

16. Direct rAAV-Mediated *In Vivo* Gene Editing of Hematopoietic Stem Cells

Ishani Dasgupta, Qiushi Tang, Terence R. Flotte, Allison
M. Keeler
Horae Gene Therapy Center, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

Gene editing of hematopoietic stem and progenitor cells (HSPCs) has progressed to clinical stage and represents a tremendously promising platform for future gene therapy for hemoglobinopathies such as sickle cell disease (Hgb SS disease). However, there are inherent practical limitations to scaling up such approaches to make them accessible to global populations most affected by these disorders. Thus, *in situ* gene modification that enables a direct targeting of the HSPCs *in vivo* would be ideal. Most prior *in vivo* editing techniques involved systemic injection of the editing machinery focused on targeting the liver, thus taking advantage of the efficiency of rAAV-mediated liver gene transfer. In this study, we are employing a targeted delivery strategy of direct injection of rAAV encoding a transgene flanked by homology arms to initiate homology directed repair (HDR) mediated gene editing of HSPCs in the bone marrow. To obtain stable transgene expression without adversely affecting endogenous gene expression, we decided to edit at the genomic safe harbor (GSH) site, AAVS1. First, we optimized the construct *in vitro* to ensure optimal transgene expression in target cells. We compared expression of the reporter GFP expressed by the CMV, EF1a and MND promoters. Human HSCs were isolated from cord blood by negative selection and enrichment of CD34+ cells, followed by electroporation with ribonucleoprotein complex containing AAVS1 guideRNA and Cas9. CD34+s cells were then transduced with rAAV6 expressing GFP from the different promoters flanked by AAVS1 homology arms. The MND promoter reported robust and long-term (up to 9 days) GFP expression in human HSCs *in vitro*. Additionally, the number of GFP expressing cells increased over time in culture suggestive of editing. Based on the *in vitro* results, we shortlisted the MND promoter for subsequent *in vivo* experiments. Since our objective is to target HSCs and cells of the hematopoietic lineage, it is necessary to assess whether the MND promoter is expressed in these cells of interest. To test this *in vivo*, firstly we established conditions for optimum engraftment and differentiation of human CD34+s in the mouse bone marrow. We then engrafted human HSCs, electroporated with CRISPR/Cas editing machinery and transduced with rAAV6 encoding GFP driven by MND promoter, flanked by AAVS1 homology arms into

immunocompromised NBSGW (nonobese diabetic (NOD)-severe combined immunodeficiency (SCID)-gamma) mice. About 40-80% human chimerism (CD45+) and multilineage distribution of HSCs was observed in peripheral blood, bone marrow and spleen of both control and transduced mice. A low frequency of GFP+ cells of the myeloid lineage was obtained in the bone marrow of the mice subjected to editing. Next, to determine HDR-based editing efficiency *in vivo*, we injected rAAV6 encoding AAVS1 homology arms and GFP driven by the MND promoter directly into the bone marrow of engrafted NBSGW mice. Our ddPCR results confirm that a localized intraosseous injection concentrates the vector in the targeted niche, thereby specifically targeting the bone marrow and enhancing transduction of the desired cell types. Future studies using a HDR based editing approach are underway to establish an optimum method for *in vivo* editing of HSPCs.

17. CRISPR-Cas9 Genome Editing of Human CD34+ Cells at Gamma-globin Promoter to Induce Fetal Hemoglobin as Sickle Cell Disease Therapy

Varun Katta[1], Kiera O'Keefe[1], Cicera R. Lazzarotto[1], Thiyagaraj Mayuranathan[1], Jonathan Yen[1], GaHyun Lee[1], Yichao Li[1], Naoya Uchida[2], Shondra M. PruettMiller[3], John Tisdale[2], Akshay Sharma[4], Mitchell J.
Weiss[1], Shengdar Q. Tsai[1]

Delivery Technologies and CRISPR for Therapeutics

Remarkably, individuals that co-inherit SCD mutations and genetic variants that cause hereditary persistence of fetal hemoglobin (HPFH) are largely asymptomatic. Thus, a promising genome editing strategy to treat SCD is to induce fetal hemoglobin (HbF, $\alpha 2\gamma 2$) to replace abnormal sickle adult hemoglobin (HbS, $\alpha 2\beta^{s}2$). Recently, disruption of an erythroid-specific enhancer of BCL11A to elevate HbF ($\alpha 2\gamma 2$) was reported to be promising in an early clinical trial (Frangoul et al.). Another promising approach is to disrupt repressor-binding motifs for BCL11A or ZBTB7A proteins in the γ-globin gene promoters in HSCs.To compare the efficiency of editing BCL11A and ZBTB7A binding sites in the γ-globin gene (*HBG1* and *HBG2*) promoters and associated levels of HbF ($\alpha 2\gamma 2$) induction, we electroporated human primary $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) with Cas9-3xNLS ribonucleoproteins (RNPs). We observed high editing efficiencies (83.8%-97.9% indels) and transplanted edited HSPCs into immunodeficient NBSGW mice. 17 weeks post-transplantation, all hematopoietic lineages derived from RNP-treated donor HSPCs exhibited 63.5 to 92.7% indel mutations at the γ-globin promoter ZBTZ7A or BCL11A binding sites, indicating consistent, high-level editing of repopulating hematopoietic stem cells (HSCs). Editing of the BCL11A binding site resulted in HbF induction up to 31.8% in erythroid progeny, compared to <2% in erythroid progeny from unedited HSCs. Disruption of the ZBTB7A binding site at similar frequencies also resulted in erythroid HbF induction, although to a lesser extent (13-18%).To assess our approach in SCD patient cells, we edited plerixafor-mobilized $CD34^+$ HSPCs from one healthy donor and three adult individuals with SCD using Cas9-3xNLS RNPs targeting the BCL11A binding site in the γ-globin promoter. We attained consistently high indel rates ranging from 80.6% to 94.5% in both $CD34^+/CD90^-$ progenitor and $CD34^+/CD90^+$ HSC-enriched populations. 17 weeks following xenotransplantation of edited cells, we observed persistent high-level editing (49.3%-91.5%) of all HSC-derived lineages with HbF levels of 18.3% to 34.3% in human erythroid progeny compared to <5% in unedited controls. Single cell western blot revealed broad HbF induction, where 49-58% of edited erythroblasts showed γ-globin expression compared to controls (<6%). One concern is that Cas9directed double strand breaks in the γ-globin promoters could result in the deletion of the intervening 4.9-kb region. By digital droplet PCR, we observed 20-25% frequencies of this 4.9 kb deletion in HSPCs prior to xenotransplantation and at 17 weeks post-engraftment. We are currently validating unintended genome wide activities identified by CHANGE-seq and *in silico* methods. In conclusion, our preclinical data suggests that *ex vivo* modification of autologous HSPCs via CRISPRCas mediated disruption of the BCL11A repressor binding site in the gamma-globin promoter genes can induce HbF to therapeutically relevant levels, and therefore, represents a promising genome editing cell therapy for SCD.

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18. In Utero Lipid Nanoparticle Delivery of CRISPR Technology to Correct Hereditary Tyrosinemia Type 1

Kshitiz Singh[1], Rachel S. Riley[2], Meghana V. Kashyap V. Kashyap[1], Brandon White[1], Sourav K. Bose[1], Haiying Li[1], Rohan Palanki[2], Margaret M. Billingsley[2], Barbara
E. Coons[1], John S. Riley[1], Philip Zoltick1[1], Kiran Musunuru[3], Michael J. Mitchell[2], William H. Peranteau[1]

[1]Center for Fetal Research, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA,[3]University of Pennsylvania, Philadelphia, PA

According to the WHO, ~295,000 newborns die within 28 days of birth every year from congenital diseases many of which have a genetic origin. Many of these diseases can be diagnosed prenatally

[1] Hematology, St Jude Children's Research Hospital, Memphis, TN,[2]Molecular and Clinical Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD,[3]Department of Cell and Molecular Biology, St Jude Children's Research Hospital, Memphis, TN,[4]Department of Bone Marrow Transplantation and Cellular Therapy, St Jude Children's Research Hospital, Memphis, TN

Sickle cell disease (SCD) affects nearly 100,000 Americans and millions of individuals worldwide. Patients with SCD are affected by pain crises, chronic anemia, multi-organ dysfunction, and early mortality.

and pathology begins before or shortly after birth. These characteristics combined with normal fetal developmental properties including the small size, an immature immune system, and an abundance of proliferating progenitor cells in multiple organs highlight the potential of *in utero* gene therapy to treat select diseases. The development of safe, clinicallyrelevant prenatal delivery methods for gene therapy, including gene editing, is critical to the future application of *in utero* gene therapy. Lipid nanoparticles (LNPs) have emerged as an alternative, potentially safer, approach for nucleic acid delivery compared to traditional viral vector delivery. In this study, we use LNPs to prenatally deliver CRISPR technology in the mouse model of hereditary tyrosinemia type 1 (HT1) in which mutations in the *Fah* gene of the tyrosine catabolic pathway result in accumulation of upstream toxic metabolites and death by ~1 month of age. Specifically, we assess LNP-mediated delivery of adenine base editor (ABE) mRNA and SpCas9 mRNA to perform liver-directed gene editing to correct the *Fah* disease-causing mutation or silence the *Hpd* gene (to prevent accumulation of toxic metabolites) respectively and rescue the lethal phenotype. In an initial experiment, gestational day (E) 16 C57BL/6 fetuses were injected via vitelline vein with LNP containing GFP mRNA. Liver analyses by immunohistochemistry (IHC) and stereomicroscope 5 days post-injection demonstrated GFP expression supporting future LNP studies in the HT1 mouse model. *Fah-/-* fetuses were via the vitelline vein at E16 with LNPs containing either ABE mRNA and a *Fah*-targeting gRNA (LNP.ABE.Fah) or SpCas9 mRNA and an *Hpd*-targeting gRNA (LNP.SpCas9.Hpd). After birth, injected fetuses were cycled off NTBC with removal of the drug by 2 weeks of age. Fetuses injected with LNPs not containing gene editing RNA molecules served as controls. On-target editing efficiency in liver DNA at the time of sacrifice (at least 90 days after removal of NTBC) was assessed by next-generation sequencing. Phenotype correction was assessed by monitoring weight gain, survival at 90 days, and liver function tests. Finally, IHC of the liver was performed to assess FAH and HPD protein expression. On-target *Hpd and Fah* editing efficiencies in liver DNA 90 days after NTBC removal were 65.51+/-1.12% and 27.01+/-0.77% respectively. The high editing efficiency 90 days after removal of NTBC in both approaches is related to a known survival advantage conferred to edited cells. IHC analysis of experimental and control mice confirmed a reduction in HPD protein expression in mice injected with LNP.SpCas9.Hpd and increased FAH protein expression in recipients of LNP.ABE.Fah. Recipients of LNP.SpCas9.Hpd and LNP.ABE.Fah demonstrated liver function, weight gain and survival that was comparable to unedited *Fah-/-* mice maintained on NTBC and significantly improved compared to unedited *Fah-/-* mice in which NTBC was removed at 2 weeks of age. This study demonstrates the feasibility of using an mRNA-LNP delivery platform for therapeutic *in utero* adenine base editing and CRISPR-mediated nonhomologous end joining. It supports this approach as a treatment for HT1 and highlights its potential to treat other genetic liver diseases.

19. Correction of DMD Mutations in Human iPS-Derived Muscle Cells by Single-Cut CRISPR/Cas9-Based Gene Editing

Ziad Al Tanoury[1], Lingjun Rao[1], Riffat Ahmed[1], Yulan Ai[1], Ben Nixon[1], Theodore Lee[1], Phoebe Tsai[1], Cristina Rodriguez Caycedo[2], Yi-Li Min[1], Eric Olson[2], Alison McVie-Wylie[1], Tudor Fulga[1]

[1]Vertex Cell and Genetic Therapies, Watertown, MA,[2]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder caused by mutations in the *DMD* gene encoding dystrophin, a protein essential for sarcolemma integrity and stability. Among more than 4,000 mutations identified in patients with DMD, the majority are deletions that cluster in "hot spot" regions. Mutations that delete single or multiple exons of the dystrophin gene can be corrected by reframing or exon skipping to restore the dystrophin open reading frame. We previously showed that single-cut CRISPR/Cas9-based gene editing can induce exon reframing or skipping events thereby restoring the synthesis of near full-length dystrophin in the skeletal muscle, diaphragm, and heart (Min et al., 2019). To validate this strategy in human cells, we used induced pluripotent stem cells (iPSCs) from DMD patients carrying dystrophin out-offrame exon deletions and from corresponding siblings encoding wild type dystrophin (non-DMD). Corrected iPSC clonal lines were derived from DMD patient cells after gene editing using *Sp*Cas9 complexed with guide RNAs targeting exons 51 and 45 (generated in Dr. Olson's laboratory at UT Southwestern). iPS cells were then differentiated into cardiomyocytes (iCM) and skeletal muscle (iSKM) cells using previously established protocols (Chal et al., 2016; Lian et al., 2012). Beating iCMs and twitching iSKMs were observed as early as 6 and 15 days, respectively. Within 3-4 weeks of differentiation, cardiac (Troponin T) and skeletal muscle (Fast MyHCII) markers were observed in differentiated cells. As expected, no dystrophin was detected in iCMs or iSKMs derived from DMD iPSCs. However, restoration of dystrophin protein expression was evident in both iCMs and iSKMs derived from corrected iPSCs. Furthermore, similar to the non-DMD derived cells, dystrophin appeared to be correctly localized at the sarcolemma on skeletal muscle fibers in all edited clones. RTPCR and Sanger sequencing confirmed exon skipping or +1 nucleotide insertions (i.e. reframing events) in iCMs differentiated from edited clonal lines. In summary, our data provides an important proof of concept for efficient correction of *DMD* mutations and restoration of dystrophin expression in human DMD patient-derived iPSCs by CRISPR/Cas9-based gene editing. Furthermore, our strategy offers an attractive platform for the development of relevant *in vitro* DMD models and drug screening strategies.

20. Cell-Based Delivery Strategies for Artificial Transcription Factors in Preclinical Animal Models

Peter Deng[1], David L. Cameron[1], Julian A. N. M. Halmai[1], Anna Adhikari[2], Nycole Copping[2], Jennifer J. Waldo[1], Jan A. Nolta[3], Jill L. Silverman[2], David J. Segal[4], Kyle D. Fink[1]

[1]Neurology, UC Davis, Sacramento, CA,[2]MIND Institute, UC Davis, Sacramento, CA,[3]Stem Cell Program, UC Davis, Sacramento, CA,[4]Biochemistry and Molecular Medicine, UC Davis, Davis, CA

Therapies based on DNA-modifying proteins such as zinc finger, transcription activator-like effectors, and CRISPR/Cas9 to regulate gene expression are becoming viable strategies to treat genetically linked disorders through manipulation of endogenous gene regulation. The identification of an effective delivery systems for these proteins *in-vivo* remain a major translational hurdle. In this work, we evaluate a mesenchymal stem/stromal cell (MSC)based delivery system as a putative cell-based strategy for the secretion of DNA modifying proteins. MSCs are advantageous as a delivery system due to their favorable ease of culturing, immunomodulatory properties, and favorable clinical safety profile. Presently, we report the first the use of a Zinc Finger secreting MSC (ZF-MSC) in transgenic Angelman Syndrome (AS) mouse models.

In our *in-vivo* work we evaluate two routes of administration for ZFMSC - direct intracranial injection or access into the cerebral spinal fluid space. Secreted ZF protein from mouse ZF-MSC is detectable in the murine hippocampus 1-week following either intracranial or cisterna magna injection. This secreted ZF is able to activate the imprinted paternal Ube3a gene in a transgenic $Ube3a^{Yfp}$ reporter mouse at 1 and 3-weeks following either intracranial or cisterna magna injection of ZF-MSC. We detect high co-localization of secreted ZF protein within the CA1 and CA3 regions of the hippocampus in the $Ube3a^{Yfp}$ reporter mouse. ZF-MSC were detectable along cerebral spinal fluid rich regions such as the lateral ventricle, 3rd ventricle, and cerebellum following either route of administration. A significant increase in Yfp+ neurons are observable 1-week following intracranial ZF-MSC administration. A significant increase in $Ube3a^{Yfp}$ protein expression is observable in the hippocampus, midbrain, and cerebellum 3-weeks following a cisterna magna injection of ZF-MSC. An amelioration of motor deficits in rotarod and forepaw propulsion is observed 3-4 weeks following intracranial injection of ZF-MSC in the $Ube3a^{mat-/pat+}$ AS mouse. Overall the results of these studies demonstrate that ZF-MSC secrete functionally active ZF protein to activate paternal Ube3a in AS mouse models. This approach may provide a less-invasive, non-surgical means to deliver gene modifying therapies into the CNS through access of the cerebral spinal fluid injections.

21. A CRISPR-Mediated Strategy for *Mecp2* Gene Correction *In Vivo* as a New Treatment for Rett Syndrome

Mirko Luoni[1], Serena Giannelli[1], Simone Bido[1], Antonio Niro[1], Eleonora Conti[1], Vania Broccoli[2]

[1] San Raffaele Scientific Institute, Milan, Italy,[2]National Research Council (CNR), Milan, Italy

Rett syndrome (RTT) is a severe neurological disorder caused by loss-of-function mutations in Methyl-CpG binding protein 2 (*MeCP2*) gene. Gene therapy strategies aimed to restore MeCP2 function hold great therapeutic potential since as shown in murine models, RTT phenotype can be reversed. Despite this, MeCP2 expression is not homogenous among different cell types and tight regulation of its expression is crucial to avoid adverse effects. Indeed, the overexpression of MeCP2 can lead to severe neurological alterations clinically comparable to RTT. In this scenario, a gene replacement approach, aimed to restore MeCP2 function under the control of the endogenous regulatory elements, represents an ideal strategy to avoid regulatory problems. Thus, we conceived a homology-independent CRISPR-based approach to introduce a minimal *Mecp2* coding sequence upstream of the endogenous translation start site. *In vitro* analyses on primary neuronal cultures revealed a high efficiency of *Mecp2* repair, with over 30% of neurons that re-activate Mecp2 expression under the control of the endogenous regulatory elements. Next, we exploited the AAVPHP.eB to validate the efficiency and the safety of this strategy *in vivo* in wild-type and symptomatic *Mecp2-null* mice. Molecular and phenotypic analyses confirmed the feasibility to repair in a site-specific manner *Mecp2* gene in the rodent brain, ameliorating the pathological phenotype of RTT mice without eliciting significant toxic reactions. This approach defines a novel therapeutic strategy with increased safety with respect to standard gene addition therapy strategies, holding important clinical implications for RTT.

Development of AAV Capsid Variants

22. A Novel Liver-Tropic AAV Capsid sL65 Shows Superior Transduction and Efficacy in Humanized Mice and Non-Human Primates

Jing Liao[1], Amy Bastille[1], Lauren Drouin[1], Matt Edwards[1], Dylan Frank[1], Laure Freland[1], Susana Gordo[1], Chih-Wei Ko[1], Noah Miller-Medzon[1], Elizabeth McCarthy[1], Nikhil Ramesh[1], Valerie Villareal[1], Jenisha Vora[1], Carmen Wu[1], Noel Walsh[1], John Xiong[1], Xiaohan Zhang[1], Shengwen Zhang[1], Marti Cabanes Creus[2], Leszek Lisowski[2], Nelson Chau[1], Kyle Chiang[1], Matthias Hebben[1]

[1]LogicBio Therapeutics, Lexington, MA,[2]Children's Medical Research Institute, The University of Sydney, Sydney, Australia

Engineering the Adeno-associated virus (AAV) capsid facilitates the generation of recombinant vectors displaying novel properties of interest, including tropism, potency, ease of production and lower recognition by preexisting antibodies. The resulting vectors could target a broader patient population and be effective at reduced dosage, with lower manufacturing cost. In addition to using cutting-edge technology to create highly diverse capsid libraries, innovative screening methods must be used to validate the libraries and to successfully identify human-tropic capsids. Previously, the Children's Medical Research Institute reported the identification of novel capsid variants exhibiting high transduction

and expression levels in human hepatocytes using a model of humanized FRG mice. Here, we further characterized these lead capsids with regards to manufacturability, seroprevalence in a panel of 200 human sera samples, and expression efficacy as measured by human Factor IX (hFIX) production in non-human primates (NHPs). Our experiments led to the identification of a novel capsid with outstanding properties related to both manufacturability and in vivo potency. When used to package a recombinant AAV genome bearing human Factor IX, this capsid sL65 produces high titers ($7x10^{11}$ vg per mL of cell culture from suspension HEK293 cells). In NHPs (*Macaca fascicularis*), the hFIX expression level delivered by sL65 was up to 4-fold higher when compared to vectors pseudotyped with benchmark capsids AAV8 and LK03. There was a tight correlation between plasma hFIX concentration, episomal copy number and mRNA level in the liver, indicating that the higher potency was induced by increased transduction and expression compared to AAV8 and LK03 capsids. Biodistribution of vectors was investigated at 6 weeks post dosing by droplet digital PCR. A strict liver tropism was observed for sL65. Interestingly, in situ hybridization staining revealed that the vector distribution was uniform in the liver while the LK03 serotype showed preferential transduction in the periportal area. None of the animals exhibited any vector-associated adverse events. Finally, we found 58% of the samples in a panel of 200 human sera had neutralizing titers < 1:10 and 67% < 1:30. In summary, these data confirm the promising potential of capsid sL65 as a new vector for human liver. With the high potency in a humanized in vivo system and non-human primates, high production yield and low prevalence of pre-existing antibodies in the human population, sL65 is an excellent candidate to overcome the current limitations of traditional AAV vectors including toxicity caused by high dosage, high manufacturing costs and low translatability from mouse studies to human trials.

23. Efficient Design of Optimized AAV Capsids Using Multi-Property Machine Learning Models Trained across Cells, Organs and Species

Shireen Abestesh, Ilke Akartuna, Alexander Brown, Megan Cramer, Farhan Damani, Jeff Gerold, Jorma Gorns, Jeff Jones, Helene Kuchwara, Jamie Kwasnieski, Sylvain Lapan, Kathy Lin, Elina Locane, Stephen Malina, Eryney Marrogi, Hanna Mendes Levitin, Patrick McDonel, Nishith Nagabhushana, Stephen Northup, Roza Ogurlu, James Oswald, Jakub Otwinowski, Chris Reardon, Chris Reardon, Cem Sengel, Amir Shanehsazzadeh, Sam Sinai, Michael Stiffler, Heikki Turunen, Flaviu Vadan, Adrian Veres, Anna Wec, Lauren Wheelock, Sam Wolock, Justin Yan,
Eric Kelsic
Dyno Therapeutics, Cambridge, MA

While next-gen high-throughput assays now enable us to learn how capsid sequence changes affect capsid functionality, measuring and optimizing capsid properties in the most therapeutically relevant models, such as non-human primates (NHP), remains challenging. The efficiency of transduction in target organs is often much lower than ideal, and most of the sequence space is non-functional. Therefore, the chance of identifying an improved variant through random search is miniscule. To overcome these challenges, we investigated to what extent machine learning models can improve the efficiency of AAV capsid design, as defined by the probability that a designed variant will have improved function. We synthesized and barcoded libraries containing 803,041 designed sequence variants of 3 natural AAV capsid serotypes and measured their properties as delivery vectors in cell culture, and in vivo in mice and NHPs. We show that single-property machine learning models trained on these data can improve the efficiency of library design by at least several hundred fold. Furthermore, we demonstrate the value of multi-property models in several ways. Models trained on multiple properties in combination help overcome data sparsity and measurement error, thereby improving model accuracy and providing a more reliable interpretation of experimental results. Multi-property models also provide a coherent framework in which to connect information from experiments across cell lines, organs, and species to the most relevant outcomes in NHP studies, thereby reducing the high resource and ethical burdens of NHP experimentation. Finally, multi-property models learn representations of the capsid landscape that can better optimize these vectors across the multiple properties that are key to enhanced therapeutic efficacy, for example: i) increasing transduction of target cells, ii) minimizing off-target tissue biodistribution, and iii) improving manufacturability. With further refinement, multi-property machine learning models will enable the design of highly optimized AAV capsids that open new frontiers in delivery, toward realizing the full potential of gene therapy.

24. Risk-Adjusted Selection for Validation of Sequences in AAV Design Using Composite Sampling

Lauren Wheelock[1], Stewart Slocum[1,2], Jorma Görns[1], Sam Sinai[1]
[1]Dyno Therapeutics, Cambridge, MA,[2]Johns Hopkins University, Baltimore, MD
High-throughput DNA synthesis methods have unlocked massive potential in the design of novel AAV vectors for gene therapy. New computational techniques are needed to generate candidate

sequences at the volume and quality necessary to take full advantage of these synthesis methods. Machine learning models trained on large datasets of AAV variants can predict the properties of unobserved sequences, allowing for screening of billions of candidates insilico before selecting the best ones for experimental validation. A naive approach to pruning billions of candidates down to hundreds of thousands for synthesis would involve selecting the top-scoring sequences according to machine learning model predictions. However, model predictions are never perfectly accurate, and a more principled approach can improve the chances of discovering high-performing variants. A similar problem arises later in the pipeline: promising candidates from high-throughput experiments are selected for medium- to lowthroughput validation by comparing their experimental measurements. Like machine learning models, experimental measurements are noisy and may be biased, and promising vectors from experiments in model organisms may not directly transfer to clinically valuable vectors in humans. Hedging against the risk of model and experimental error in this context is critical for improving the chances that the validation set includes the variants with the most desired ground-truth properties. Here we demonstrate the utility of a novel method, Composite Sampling (CS), that allows for pruning candidate sequences by accounting for model uncertainty and experimental noise, in a manner that maximizes the chances of including the best candidates in our validation set. CS is a tunable computational method that allows the experimenter to modulate validation risk when faced with noisy model or experimental evaluation. This method can be used in any setting where samples can be scored according to a desirability and a diversity metric. Here we show our method's value for AAV capsid design. We use retrospective studies in experimental AAV datasets and simulation of multiple synthetic datasets to demonstrate that Composite Sampling is consistently better than greedy approaches in selecting highperforming sequences for the validation set. In comparison to current approaches, our method increases the probability that high-throughput screens of capsid libraries will yield vectors that are optimized for therapeutic applications.

25. A Comparison of Methods Used for the Determination of Full and Empty rAAV Particles

Bryan Troxell[1], I-Wei Tsai[1], Mark Rodgers[1], Kevin Mouillesseaux[2], Jack Jingi Ren[3]

[1]Analytical Development and Research Quality Control, StrideBio, Inc, Morrisville, NC,[2]Process Development, StrideBio, Inc, Research Triangle Park, NC,[3]Research Vector Production, StrideBio, Inc, Research Triangle Park, NC

The use of recombinant Adeno-associated virus (rAAV) as a biological vehicle for the delivery of therapeutic DNA into humans provides an unparalleled treatment for a range of diseases. Although the production of therapeutic biologics for treating human diseases has been established, the first FDA approved gene therapy for human use occurred within recent years. To produce rAAV at the appropriate scale for treatment, requires effective manufacturing and purification of capsids containing the target DNA from the complex matrix of the upstream harvest. These processes may introduce undesirable process and product-related impurities that impact the overall performance and quality of the final drug product. One such product-related impurity is the presence of improperly packaged or "empty" (hereafter referred to as empty) rAAV capsids. The collective data indicate that the level of empty capsids in final products may reduce the *in vivo* efficacy and result in adverse events following administration. Regulatory guidance suggests that accurate analytical methods be used early in the product development cycle of gene therapies to evaluate and monitor the level of empty capsids. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is considered a primary analytical method for the determination of full and empty rAAV particles. Additional methods have been utilized for the determination of the full empty ratio. These methods include transmission electron microscopy (TEM), vector genome to capsid determinations, and more recently cryogenic electron microscopy (Cryo-EM) and charge detection mass spectrometry (CD-MS). Each of these methods offers advantages and disadvantages in terms of assay sensitivity, accuracy, linearity, robustness, and utility for in-process testing. Presented here is the comparison of these methods (notably SV-AUC and CD-MS)

with an internally developed method to assess the full-to-empty ratio. A variety of parental and engineered proprietary capsids containing single-stranded and self-complementary vectors were assessed. Across multiple productions, SV-AUC, CD-MS, and our internal method were in good agreement for a variety of packaged DNA targets. SV-AUC and CD-MS are especially advantageous for the quantification of partially packaged DNA in rAAV, which tends to increase in abundance with self-complementary vectors. The internal method allowed for the timely measurement of full-to-empty particles at the cell lysate stage as well as purified material; however, the stage of sample purification as well as the packaged genome may impact which method is most suitable for determining levels of empty, partial, and full capsids. Results from this work suggest there is a benefit to using orthogonal methods to determine the level of empty particles.

26. A Tetracycline Enabled Self-Silencing Adenovirus (TESSA) Platform Delivers High-Quality, High-Titre, Multi-Serotype Recombinant Adeno-Associated Virus (AAV) Stocks

Maria Patricio, Weiheng Su, Qian Liu, David W. Brighty, Ryan Cawood

Oxgene, Oxford, United Kingdom

A significant challenge for Adeno-associated virus (AAV) -mediated gene transfer has been the capacity to produce recombinant AAV vectors of high quality, at high-titre and at large scale. We have addressed these challenges by developing a proprietary TetracyclineEnabled Self-Silencing Adenovirus (TESSA) system that simultaneously provides Adenovirus (Ad) helper functions and encoded AAV Cap/ Rep. When cells carrying an AAV ITR-flanked transducing genome are infected with TESSA-Cap/Rep, high-titre rAAV stocks are produced that are essentially free of contaminating TESSA vector. We have now examined the ability of TESSA to

support production of adiverse range of AAV serotypes. The Capcoding region of AAV serotypes 1-9 were independently engineered into the E1 region of TESSA and the quality, physical and infectious titre of the rAAV produced by this system were characterised. For all rAAV serotypes, physical titres and infectious titres were invariably greater than that observed for conventional plasmid-based rAAV systems. For these serotypes, the ratio of full to empty particles is greatly improved. Moreover, for some AAV serotypes, the TESSA system delivered infectious titres 1000-fold greater than the plasmid-based helper-free systems. Importantly, TESSA-derived rAAV serotypes are competent for reporter gene and therapeutic gene transfer into a multiplicity of human cell types in vitro and a variety of model animal tissues in vivo. Therefore, we have developed a versatile system that can be applied to a range of naturally occurring AAV serotypes, and to experimentally engineered AAV capsids with novel tissue targeting phenotypes. The TESSA system provides unique opportunities for robust high-quality scale up of rAAV manufacture that will reduce costs and facilitate rapid progression to clinical application of novel rAAV-based gene therapies.

27. Next Generation AAV Drug Products: Enhanced Stability & Clinical Ease for High Titer Preparations

Lori B. Karpes, Tyler J. Peters, Mark Bailey, Michael Mercaldi, Eric Faulkner, Tim Kelly
Homology Medicines, Bedford, MA

Homology Medicines has developed a Plug-&-Play Process and Manufacturing Platform to develop and manufacture gene transfer and gene editing therapeutics for rare diseases, which utilizes our proprietary Clade F AAV capsids derived from hematopoietic stem cells (AAVHSCs). As our programs have progressed through development and into the clinic, we have increased focus on the Drug Product sciences which includes the thoughtful development of stabilizing formulations enabling high titers, easing of clinical storage and supply chains, and enhanced long-term stability. AAV preparations have long held a reputation as challenging not only for production, but for long-term stability at even low concentrations. However, here we demonstrate not only the stability of AAVHSCs in the liquid state, but also the impact of novel formulations on capsid stability. Our AAV preparations achieve titers in excess of 1E14 vg/mL and demonstrate stability for a minimum of one year at 2-8°C and more than six months at room temperature. Benefits of vector stability in the liquid state are a reduction in the need for -80°C-storage infrastructure and simplification of the clinical supply chain by enabling 2-8°C storage at the manufacturing site and clinical pharmacy. This work demonstrates the marked stability of our proprietary AAVHSCs as well as the impact of a well-developed formulation. Furthermore, this effort shows that by bringing a Drug Product focus to AAV product development, an organization can help support clinical and commercial success by providing long product expiries, smaller administration volumes, and 2-8°C supply chains.

28. Development of a Membrane-Based Affinity Matrix for Downstream Purification Process of Adeno-Associated Virus Vectors

Takuma Sueoka, Masakatsu Nishihachijo, Hisako Yaura, Shota Hirayama, Masahiro Aratake, Hiroyuki Watanabe, Kazunobu Minakuchi
KANEKA Corporation, Takasago, Hyogo, Japan

Adeno-associated virus (AAV) vectors are promising tools for gene therapy. Along with expanding market scale, reducing production costs is a key concern. Although the titer of AAV vectors in the upstream process has increased over the years, the downstream process is still outdated and should be improved. Affinity chromatography is a powerful tool for capture step of biologics production owing to its high selectivity. However, the chromatographic purification of AAV vectors from crude cell lysate is challenging due to high impurity contents in the loading material and damage to AAV transduction efficiency by contacting with low pH solution in the elution step. Tangential-flow filtration (TFF) is introduced prior to affinity chromatography to reduce the burden and to concentrate AAV vectors, but introduction of an additional process is ideally avoided to simplify the process and reduce production costs. Preventing damage from low pH denaturation increases overall AAV vector productivity in terms of a yield and quality. Herein, we developed a novel affinity matrix, which is composed of membrane-based matrix with a larger pore size than conventional beads resin. This enables a higher flow rate, resulting in a ten-fold shorter contact time than the conventional resin that compensates for the lack of the concentrating effect of TFF. This matrix provided a higher AAV vector yield in the elution pool than the beads resin owing to less contact with low pH conditions. Moreover, better cleanability was also shown likely due to less clogging inside the large pores. Our new affinity matrix intensifies the AAV vector affinity chromatography step and shows insight to develop a new platform of AAV vector downstream process.

Gene Therapy for Inborn Errors of Metabolism

29. Coadministration of AAV Expressing MDR3 (VTX-803) and ImmTOR Allows for Vector Re-Administration to Treat Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) in Juvenile *Abcb4*$^{-/-}$ Mice

Nicholas D. Weber[1], David Salas-Gómez[2], Leticia Odriozola[2], Irene Ros-Gañán[1], Mirja Hommel[2], Takashi Kei Kishimoto[3], Jean-Philippe Combal[4], Gloria González-Aseguinolaza[2,5,6]
[1]Vivet Therapeutics S.L., Pamplona, Spain,[2]Division of Gene Therapy and Regulation of Gene Expression, Cima Universidad de Navarra, Pamplona,

Spain,[3]Selecta Biosciences, Watertown, MA,[4]Vivet Therapeutics S.A.S., Paris, France,[5]Vivet Therapeutics, Pamplona, Spain,[6]Instituto de Investigación Sanitaria de Navarra (IdISNA), Pamplona, Spain

Liver-directed AAV gene therapy has been shown to be an effective modality for the correction of genetic disorders, such as hemophilia, in adult patients. However, gene therapy for many inborn errors of metabolism, such as progressive familial intrahepatic cholestasis type 3 (PFIC3), would be most effective if administered in infancy or early childhood, to prevent irreversible damage. PFIC3 is a rare monogenic disease leading to cholestasis, cirrhosis and ultimately liver failure, with generally an early onset that requires early treatment when diagnosed in children under 3 years of age. PFIC3 is characterized by a dramatic reduction in biliary phosphatidylcholine (PC) content due to mutations in the *ABCB4* gene, which codes for multidrug resistance protein 3 (MDR3) and is responsible for transporting PC across the canaliculi membranes of hepatocytes into the bile, where PC neutralizes bile acid toxicity. A key challenge for AAV gene therapy in pediatric PFIC3 patients is the potential for therapeutic benefit to wane over time as the non-replicating AAV vector is diluted due to hepatic cell proliferation in the growing child, and that AAV cannot be re-administered due to the formation of high titers of persistent neutralizing antibodies. Here we tested the effectiveness of tolerogenic ImmTOR nanoparticles carrying the immunomodulating drug rapamycin to enable repeated intravenous administration of an hepatotropic AAV vector carrying human *ABCB4* cDNA (VTX-803) in 2-week-old juvenile *Abcb4*$^{-/-}$ mice when ImmTOR is co-administered with the first treatment of AAV. As a proof-of-concept study, we utilized a subtherapeutic dose of VTX-803 in order to focus on the experimental outcomes of coadministration with ImmTOR and readministration. VTX-803 when co-injected with ImmTOR allowed for a successful readministration of VTX-803 alone two weeks later and resulted in a robust and stable correction of the disease phenotype lasting over 7 months, while repeat dosing of the vector alone (at a sub-therapeutic dose) or together with empty SVP did not exhibit a therapeutic effect. In males, a temporary therapeutic effect was observed following only a single treatment of VTX-803 with ImmTOR, but not observed with empty SVP, suggesting that ImmTOR could increase vector transduction and/or transgene expression. This effect was observed through 10 weeks post-treatment, after which time the effect was lost, highlighting the importance of repeat dosing especially in juvenile animals with growing livers where transgene-positive cells can be diluted over time. Vector treatment without ImmTOR resulted in the production of AAV-specific neutralizing antibodies (NAbs), while vector coadministration with ImmTOR prevented NAbs production. Thus, this provides further evidence that redosing AAV gene therapy can be achieved through coadministration with ImmTOR, which has the dual benefit of potentially improving AAV vector transduction and mitigating vector-specific immunogenicity.

30. Preclinical Evaluation of Combined Adeno-Associated Virus and Nanoparticle Delivery of piggyBac® Transposon System for Durable Transgene Expression in the Growing Neonatal Murine Liver

Jingjing Jiang, Bernard Kok, Xinggang Liu, Vananh Pham, David Ebeid, Mehul Dhanani, Sean Essex, Jivan Yewle, Brian Truong, Devon J. Shedlock, Joshua Rychak, Bruce F. Scharschmidt, Eric M. Ostertag, Julian D. Down

Poseida Therapeutics, San Diego, CA

Gene delivery via recombinant adeno-associated virus (rAAV) has been shown to be efficacious in pre-clinical models and clinical trials for a variety of genetic diseases. A major limitation of current rAAVmediated gene therapy, however, is dilution of episomal rAAV and loss of transgene expression in rapidly dividing tissues as well as toxicity at higher rAAV doses. Loss of transgene expression and therapeutic efficacy are particularly limiting for gene therapy of infants and young children severely affected by metabolic and other disorders involving the liver. To address these issues, we have explored rAAV and/or novel nanoparticles (NP) as vectors for delivery of piggyBac® transposon and transposase to facilitate transgene integration in the host hepatocyte genome using ornithine transcarbamylase (OTC) deficiency as a disease model. A human OTC expression cassette in a piggyBac® transposon with liver-specific promoters was administered using rAAV with a liver-tropic capsid to neonatal wild-type and OTC deficient (Spf^{ash}) mice, with and without "Super" piggyBac (SPB), a hyperactive form of the transposase. As compared with transposon alone, concomitant rAAV delivery of SPB resulted in stable vector integration into the hepatocyte genome with durable and enhanced transgene expression for over 3 months according to vector copy number, bioluminescence imaging, IHC, human OTC mRNA and

protein levels and mitigation of the OTC disease phenotype. We further demonstrated that replacing rAAV delivery of SPB with a NP formulation for efficient liver delivery of mRNA resulted in similar high levels of durable transgene expression (~30% hepatocytes). Genomic modification and clonality of transposed hepatocytes are being further evaluated from LM-PCR and integration site analysis. These findings collectively demonstrate the unique potential of piggyBac® technology for *in vivo* liver-directed gene therapy for infants and young children, as well as the versatility afforded by utilizing viral and/or non-viral delivery systems for single-treatment and permanent correction of OTC deficiency and other genetic diseases.

31. Targeting Aberrant Acylation as a Novel Approach for Treating Methylmalonic Acidemia (MMA) and Related Other Organic Acidemias

Sangho S. Myung[1], PamelaSara Head[2], Jessica L. Schneller[1], Samantha McCoy[1], Yong Chen[3], Marjan Gucek[3], Irini Manoli[1], Charles P. Venditti[1]

[1]NHGRI, NIH, Bethesda, MD,[2]NIGMS, NIH, Bethesda, MD,[3]NHLBI, NIH, Bethesda, MD

Organic acidemias (OAs), such as methylmalonic acidemia (MMA), are a group of clinically severe inborn errors of metabolism that typically arise from defects in the catabolism of amino- and fatty acids. The accretion of acyl-CoA species is postulated to cause intracellular toxicity and underlie the dysregulation of multiple intermediary pathways seen in the patients, such as the urea cycle and glycine cleavage system. Here, we explore an alternative pathophysiological consequence of impaired acyl-CoA metabolism: the accumulation of aberrant posttranslational modifications (PTMs) on enzymes in critical intracellular pathways. Using an MMA mouse model that recapitulates MMA-associated hepatic mitochondriopathy (Mmut-/-;TgMCKMmut), we surveyed PTMs in hepatic extracts with acyl-lysine antibodies and discovered widespread hyper-acylation. Next, we prepared affinity anti-acyl-lysine columns to enrich for modified proteins, and then performed mass spectrometry to characterize the PTM proteome. Excessive acylation of enzymes involved in glutathione, urea, arginine, tryptophan, valine, isoleucine, methionine, threonine, and fatty acid metabolism were detected in the MMA mice, and validated via immunoprecipitation analysis. We extended our analyses to other perturbed pathways, including the glycine cleavage system and mitochondrial replication, which we found to also be aberrantly modified in liver extracts from both MMA patients and Mmut-/-;TgMCKMmut mice as compared to respective controls The emerging pattern from our aggregate studies further supports a model where hyperacylation of key enzymes in pathways known to be dysregulated in MMA likely contributes to altered metabolism and identifies a new set of targets. With these new insights, we investigated the sirtuin (SIRT) family of enzymes as potential therapeutic agents given their known role as specialized deacylases. SIRT 1-7 were individually assayed for activity toward MMA specific acylations of modified protein substrate. An optimal SIRT emerged, but we noted that aberrant acylation also inhibited the functionality of our candidate enzyme, lowering enzymatic activity *in vitro.* Using rational mutagenesis, we created a "SuperSIRT" that was resistant to acylation-dependent inhibition, validated activity in vitro, cloned it behind a liver specific promoter (LSP), and packaged

with an AAV8 capsid. The resulting AAV8 LSP SuperSIRT or an AAV8 LSP EGFP control were then systemically delivered to juvenile Mmut-/-;TgMCKMmut and Mmut+/-;TgMCKMmut mice at a dose of $1e^{13}$ GC/kg, and followed by clinical, biochemical, and enzymatic analyses. After 1 month, the blood ammonia levels were significantly reduced in the treated mutant mice compared to the AAV8 LSP EGFP and untreated mutant control groups, while plasma methylmalonic acid levels remained unchanged. In hepatic extracts from the AAV8 LSP SuperSIRT treated Mmut-/-;TgMCKMmut mice, the aberrant acylation on key protein targets in the urea cycle and glycine cleavage pathway was reversed compared to GFP treated controls. In summary, our studies have identified a new PTM axis in patients and mice with MMA, which has allowed the development of a SuperSIRT gene therapy that could be used to treat all forms of MMA and might be extended to other disorders where aberrant acylation plays a role in disease pathophysiology, such as organic acidemias and fatty acid oxidation disorders.

32. AAV Liver Gene Therapy-Mediated Inhibition of FGF23 Signaling as a Therapeutic Strategy for X-linked Hypophosphatemia

Louisa Jauze[1], Volha Zhukouskaya[1], Severine Charles[1], Christian Leborgne[1], Agnes Linglart[2], Catherine Chaussain[3], Claire Bardet[3], Giuseppe Ronzitti[1]

[1]Genethon, Evry, France,[2]Univesite Paris Saclay APHP, Le Kremlin-Bicêtre, France,[3]Université de Paris, Montrouge, France

X-linked hypophosphatemia (XLH) is a rare disease due to increased fibroblast growth factor 23 (FGF23) secretion from bones which results in phosphate wasting in kidneys. Decreased circulating phosphate is the primary cause of severe skeletal deformities and short stature that greatly affect patients' quality of life. Conventional treatment for XLH requires life-long, repeated supplementation of phosphate and active vitamin D analogs and is associated with severe long-term side effects and poor compliance. Recently, the use of Burosumab (Crysvita®, Ultragenyx, and Kyowa Kirin), a monoclonal antibody for FGF23, has been proposed as an alternative treatment. Based on the central role of the overactive FGF23 pathway in the pathophysiology of FGF23, here we devised a liver-targeted AAV gene therapy strategy to inhibit FGF23 signaling and rescue bone pathology with a single injection. Secretion of an FGF23 competing factor (cFGF23) by the liver of a murine model of XLH led to the restoration of the impaired skeletal phenotype, significant reduction of osteomalacia and bone and joint alterations in Hyp-Duk mice. Our data provide proof-of-concept to the use of AAV liver gene therapy for the treatment of XLH, a prototypical disease associated to overexpression of soluble factors in tissues refractory to AAV gene therapy, thus expanding the reach of this therapeutic modality and providing novel options for the treatment of this disease category.

33. Comparison of Gene Addition Therapy in Genetically Distinct Mouse Models of Classical Phenylketonuria

Daelyn Y. Richards[1,2], Michael A. Martinez[1], Shelley R.

Winn[1], Sandra Dudley[1], Cary O. Harding[1]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]Department of Medical Education and Clinical Sciences, Washington State University Elson S. Floyd College of Medicine, Spokane, WA

Phenylketonuria (PKU) is a highly complex biochemical disorder with nearly 1,000 pathogenic alleles in the phenylalanine hydroxylase (*PAH*) gene that cause a wide spectrum of effects on PAH enzyme dynamics. Clinical trials examining gene addition therapy for classical phenylketonuria (cPKU) have launched with much excitement, yet there is a lack of preclinical data on therapeutic outcomes in genetically distinct animal models. The longstanding cPKU $Pah^{enu2/enu2}$ mouse contains a missense mutation p.F263S (c.835 T > C), resulting in normal production of aberrant non-functional PAH. It has long been hypothesized that the mutant PAH monomers in this model may interfere with wildtype (WT) monomers, impairing overall function of the PAH holoenzyme complex, a phenomenon known as the dominant negative effect. Recently we created the $Pah^{dexon1/dexon1}$ cPKU mouse that contains a deletion of *Pah*

exon1 and is completely void of PAH protein, thus removing the variable of mutant monomer expression. In comparing overall PAH enzyme activity in heterozygous $Pah^{+/enu2}$ (N=6) and $Pah^{+/dexon1}$ (N=3) animals, we found that $Pah^{+/enu2}$ animals revealed markedly decreased enzyme activity (29 ± 5% WT activity) in comparison to $Pah^{+/dexon1}$ animals (58 ± 7% WT activity) with a $P < 0.0001$, and for the first time, indisputably confirmed the presence of a dominant negative effect caused by the mutant monomers produced from the Pah^{enu2} allele. This was further supported with liver-directed gene addition therapy studies comparing the two models. The murine liver-tropic adeno-associated virus serotype 8 was packaged with a Liver Specific Promoter driving expression of murine PAH (AAV2/8 LSPmPAH) and administered to 6-8 week old animals via retro-orbital injection that were harvested after two weeks for molecular analyses. All animals showed corrected blood Phe well below the therapeutic target range (below 360 μM), however they revealed stark differences in total PAH enzyme activity between $Pah^{enu2/enu2}$ and $Pah^{dexon1/dexon1}$ cPKU animals. Two $Pah^{enu2/enu2}$ cohorts of 3 males and 3 females (N=6 each) received medium dose (1×10^{11} vector genomes [vg]) or high dose (1×10^{12} vg) gene therapy. The total vector genomes per diploid liver genome (vg/dlg) ranged between 8-42 vg/dlg and 56-291 vg/ dlg, respectively, resulting in enzyme activity of 7.7 ± 2.2% and 14.8 ± 3.3%, respectively. In single $Pah^{dexon1/dexon1}$ animals, low dose (1×10^{10} vg), medium dose (1×10^{11} vg) and high dose (1×10^{12} vg) gene therapy conferred total liver vector genome copies of 2.9 vg/dlg, 16.2 vg/dlg and 50 vg/dlg, which resulted in an astonishing 4.8%, 42%, and 96.1% WT PAH enzyme activity. While more studies are needed to include more $Pah^{dexon1/dexon1}$ animals at all doses and $Pah^{enu2/enu2}$ animals at low doses, this data reveals the profound impacts genotype can have on overall enzyme dynamics in the setting of gene addition therapy for PKU. A further advantage of the $Pah^{dexon1/dexon1}$ cPKU animal model for gene therapy development is the ability to perform immune-based molecular analyses to localize and quantify gene therapy delivered PAH enzyme, a feat that is impossible in the $Pah^{enu2/enu2}$ model. Liver immunohistology of gene therapy treated $Pah^{dexon1/dexon1}$ animals revealed a dose dependent expression of PAH, which reveals critical knowledge that could be used to further optimize gene therapy development for PKU.

34. AAV8 Gene Therapy as a Potential Treatment in Adults with Late-Onset Ornithine Transcarbamylase (OTC) Deficiency: Updated Results from a Phase 1/2 Clinical Trial

Cary O. Harding[1], Maria Luz Couce[2], Tarekegn Geberhiwot[3], Wen-Hann Tan[4], Aneal Khan[5], Luis Aldamiz-Echevarria[6], George A. Diaz[7], Connie Lee[8], Ana Cristina Puga[8], Eric Crombez[8]

[1]Oregon Health & Science University, Portland, OR,[2]University of Santiago de Compostela, Santiago de Compostela, Spain,[3]University of Birmingham, Birmingham, United Kingdom,[4]Boston Children's Hospital, Harvard Medical School, Boston, MA,[5]University of Calgary, Calgary, AB, Canada,[6]Cruces University Hospital, Biocruces Bizkaia Health Research Institute, Barakaldo, Spain,[7]Icahn School of Medicine at Mount Sinai, New York, NY,[8]Ultragenyx Gene Therapy, Cambridge, MA

Introduction: OTC deficiency is an X-linked urea cycle disorder resulting in episodic hyperammonemia that can cause cumulative neurocognitive damage and even death. The current standard of care includes a proteinrestricted diet and nitrogen-scavenging agents, but there remains high unmet medical need with continued risk of hyperammonemic crises. DTX301, an AAV8 vector containing the *OTC* transgene, is currently under investigation for the treatment of OTC deficiency. **Methods:** CAPtivate (NCT02991144) is an ongoing global, multicenter, open-label phase 1/2 dose-escalation trial evaluating the safety and preliminary efficacy of DTX301 in adults with late-onset OTC deficiency. The primary endpoint is incidence of adverse events (AEs). Secondary endpoints are changes in the rate of ureagenesis and 24-hour plasma ammonia levels. Patients received a single IV infusion of DTX301 at the following doses: Cohort 1 (2×10^{12} Genome Copies [GC]/kg), Cohort 2 (6×10^{12} GC/kg), and Cohort 3 (1×10^{13} GC/kg). Study duration is 52 weeks followed by up to 4 years of long-term follow up. A partial responder is defined as a patient with a clinically meaningful and sustained increase in rate of ureagenesis with stabilization or improvement in ammonia control. A complete responder is defined as a patient who has also successfully discontinued ammonia-scavenging drugs and protein-restricted diet. **Results:** DTX301 dosing of 3 patients in each of cohorts 1, 2, and 3 is complete. Cohort 4 (1×10^{13} GC/kg with prophylactic oral steroid taper) is enrolling. No treatment-related serious AEs or dose-limiting toxicities were reported; all AEs were mild or moderate (grade 1, 2) during the study. Seven patients experienced treatment-emergent AEs (TEAEs) that were considered related to study drug. Five patients experienced asymptomatic ALT increases consistent with those seen in other AAV gene transfer clinical trials. ALT increases were managed and resolved with a protocol-specified tapering regimen of oral corticosteroids administered in outpatient setting. Other TEAEs considered related to study drug were photophobia, headache, hypertension, vector-induced hepatitis, and hypophosphatemia. Overall, 6 of 9 patients responded to DTX301: 3 patients were complete responders, and 3 patients were partial responders. All 9 treated patients maintained or improved ammonia control. Cohort 1 had one complete responder. Cohort 2 had 1 complete responder and 1 partial responder. Cohort 3 had 1 complete responder and 2 partial responders. The longest-treated responders from cohorts 1 and 2 are showing a durable response at 2.5 to 3 years after treatment and remain clinically and metabolically stable with good ammonia control. **Conclusions:** Data from CAPtivate indicate that DTX301 has an acceptable safety profile and may be a potential new therapy with longterm therapeutic benefit for patients with OTC deficiency. Followup of all patients is ongoing and enrollment in cohort 4 is nearly complete.

35. AAV-Mediated Delivery of MiRNA-34B/C Improves Liver Fibrosis

Pasquale Piccolo[1,2], Rosa Ferriero[1], Anna Barbato[1],

Sergio Attanasio[1], Marcello Monti[1], Claudia Perna[1], Florie Borel[3], Patrizia Annunziata[1], Annamaria Carissimo[1], Rossella De Cegli[1], Severo Campione[4], Luca Quagliata[5], Luigi Terracciano[5], Chantal Housset[6,7], Jeffrey H. Teckman[8], Christian Mueller[3], Nicola Brunetti-Pierri[1,2]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy,[2]Department of Translational Medicine, Federico II University, Naples, Italy,[3]Department of Pediatrics and Horae Gene Therapy Center, UMass Medical School, Worcester, MA,[4]Pathology Unit, Cardarelli Hospital, Naples, Italy,[5]Molecular Pathology Division, Institute of Pathology, University of Basel, Basel, Switzerland,[6]Centre de Recherche Saint-Antoine, Sorbonne Université, INSERM, Paris, France,[7]Department of Hepatology, CRMIVB-H, Saint-Antoine Hospital, Assistance Publique-Hôpitaux de Paris, Paris, France,[8]St. Louis University School of Medicine, Cardinal Glennon Children's Medical Center, St. Louis, MO

Liver fibrosis is a major complication of chronic liver diseases and is orchestrated by an intricate molecular network. MiRNAs have been known to regulate several distinct processes involved in liver fibrosis. In this study, we found a novel miRNA cluster, including miR-34b and -c, to be upregulated in several forms of liver fibrosis both genetic and acquired. We found that murine hepatic miR-34b/c is upregulated in liver diseases due to ABCB4 deficiency and α-1 antitrypsin deficiency, disorders prone to the development of liver fibrosis. miR-34b/c upregulation was also found to occur in mouse models of liver fibrosis induced by thioacetamide and CCl_4. Mechanistically, miR-34b/c expression was dependent upon JNK-mediated phosphorylation of FOXO3 transcription factor on Ser574. Deletion of miR-34b/c resulted in early development of liver fibrosis and increased signaling of PDGF, a target of miR-34b/c. Moreover, miR-34b/c was effective in blunting TGF-β-mediated activation of human hepatic stellate cells, a key event in liver fibrosis development. Finally, we found that AAVmediated hepatocyte-specific overexpression of miR-34b/c significantly ameliorated liver fibrosis in two independent mouse models of acquired liver fibrosis (thioacetamide and CCl_4).In conclusion, this study reveals a novel pathway involved in liver fibrosis that is potentially implicated in both genetic and acquired forms of hepatic fibrosis. Furthermore, it supports miR-34b/c as a novel therapy against liver fibrosis.

Genetic Blood and Immune Disorders

Genetic Blood and Immune Disorders

36. Follow-Up of a Phase I/II Gene Therapy Trial in Patients with Fanconi Anemia, Subtype A

Julian Sevilla[1], Paula Rio[2], Susana Navarro[2], Rebeca Sánchez-Domínguez[2], Jose C. Segovia[2], Wei Wang[3], Josune Zubicaray[1], Rosa Yañez[2], Jose A. Casado[2], Yari Gimenez[2], Francisco J. Roman-Rodriguez[2], Omaira Alberquilla[2], Eva Galvez[1], Eva Merino[1], Jordi Barquinero[4], Anne Galy[5], Nagore Garcia de Andoin[6], Ricardo Lopez[7], Albert Catala[8], Francois Lefrere[9], Marina Cavazzana[10], Gayatri Rao[11], Jonathan Schwartz[11], Roser M. Pujol[12], Jordi Surralles[12], Jean Soulier[13], Manfred Schmidt[3], Cristina Diaz de Heredia[4], Juan Bueren[2]

[1]Servicio Hemato-Oncología Pediátrica, Fundacion Biomédica Hospital Niño Jesús, Madrid, Spain,[2]Hematopoietic Innovative Therapies, CIEMAT/CIBERER/ IIS-FJD, UAM, Madrid, Spain,[3]GeneWerk, GmbH, Heidelberg, Germany,[4]Hospital Val d'Hebron, Barcelona, Spain,[5]Genethon, Evry, France,[6]Hospital Universitario de Donostia, San Sebastian, Spain,[7]Hospital de Cruces, Bilbao, Spain,[8]Hospital San Joan de Deu, Barcelona, Spain,[9]Hopital Necker-Enfants Malades, Pari, France,[10]Hopital Necker-Enfants Malades, Paris, France,[11]Rocket Pharmaceuticals, Inc., New York, NY,[12]Servicio de Genética e Institut de Reserca / Departmento de Genética y Microbiología, IIB-Sant Pau, Hospital Sant Pau/ Universitat Autònoma de Barcelona/CIBERER, Barcelona, Spain,[13]Hôpital Saint-Louis and University Paris Diderot, Paris, France

Fanconi anemia (FA) is a monogenic inherited disorder mainly characterized by congenital abnormalities, childhood bone marrow failure (BMF) and cancer predisposition. Here we report the results of the 1-3 year follow-up of the eight evaluable FA-A patients corresponding to a phase I/II gene therapy trial. $CD34^+$ cells were mobilized with filgrastim and plerifaxor and collected following 2-3 apheresis procedures. $CD34^+$ cell-enriched fractions were transduced during a total period of 20-24h with the PGK-FANCA.Wpre* lentiviral vector and then infused without any pre-conditioning regimen. Nine patients age 3-7 years were infused with a range of $7.3x10^4$ to $1.9x10^6$ $CD34^+$ cells/kg. One patient was withdrawn from the clinical trial due to contamination of the medicinal product and bacteremia which was treated by antimicrobial therapy. Vector copy numbers (VCN) in colonies derived from the manufacturing products ranged from 0.2 to 0.9 VCN/cell. VCNs in patients' PB and BM after infusion of transduced cells showed slow but progressive engraftment of genecorrected cells in six out of the eight evaluable patients, reaching stabilized values as high as 0.6 copies/cell through the end of the follow-up (3 years). Insertion site studies confirmed the safety of the LV with 1-3 years follow-up. VCN increases were associated both with a progressive MMC-resistance of BM progenitor cells and a reduction in the chromosomal instability in PB T cells exposed to diepoxybutane. Stabilized, and even improved PB cell counts have been observed in patients with higher levels of gene correction. Nevertheless, two out of the eight evaluable patients who were treated at advanced stages of the disease or infused with low numbers of corrected $CD34^+$ cells showed progressive evolution of BMF. Our results demonstrate that gene therapy of non-conditioned FA patients has the potential to prevent BMF, and support the infusion of corrected $CD34^+$ cells prior to the development of BMF. Based on these results, a global phase II clinical trial is currently ongoing under the sponsorship of Rocket Pharmaceuticals Inc., focused on the prevention of BMF by means of the infusion of higher numbers of corrected $CD34^+$ cells in early stages of the disease.

37. Liver Gene Therapy with Lentiviral Vectors Corrects Hemophilia A in Mice and

Achieves Normal-Range Factor VIII Activity in Non-Human Primates

Michela Milani[1], Cesare Canepari[1,2], Tongyao Liu[3], Mauro Biffi[1], Fabio Russo[1], Tiziana Plati[1], Rosalia Curto[1], Susannah Patarroyo-White[3], Ilaria Visigalli[1], Paola Albertini[1], Eduard Ayuso[4], Christian Mueller[3], Andrea Annoni[1], Luigi Naldini[1,2], Alessio Cantore[1,2]

[1]San Raffaele-Telethon Institute for Gene Therapy, Milano, Italy,[2]Vita Salute San Raffaele University, Milano, Italy,[3]Sanofi, Waltham, MA,[4]INSERM UMR1089, University of Nantes, Nantes, France

Liver gene therapy with adeno-associated viral (AAV) vectors delivering a clotting factor transgene into hepatocytes has shown multiyear therapeutic benefit in adults with hemophilia. However, the mostly episomal nature of AAV vectors currently challenges application of AAV-vector mediated liver gene therapy to young pediatric patients. In contrast, lentiviral vectors (LV) integrate into the target cell chromatin and are maintained as cells divide. We previously developed LV that achieve stable and therapeutic levels of coagulation factor IX (FIX) transgene expression in the liver of adult mice, dogs and non-human primates (NHP) after systemic delivery. To evaluate LV-mediated liver gene therapy for hemophilia A, we generated LV expressing engineered versions of the human B-domain deleted Factor VIII (FVIII) by codon optimization of the transgene (coFVIII) and the inclusion of a nonstructured XTEN polypeptide (coFVIII.XTEN), known to increase the half-life and secretion of the payload protein, in the B-domain region. We administered LV expressing FVIII, coFVIII or coFVIII. XTEN intravenously (i.v.) to newborn hemophilia A mice and observed long term FVIII activity up to 200% of normal and restoration of hemostasis in mice treated with LV encoding for engineered transgenes, with transgene output 10-20 fold higher for coFVIII compared to FVIII transgene and 10-fold higher for coFVIII.XTEN than coFVIII. We then set out to evaluate FVIII expression in NHP and produced large-scale batches of allo-antigen free and phagocytosis shielded (CD47 high) LV carrying coFVIII or coFVIII.XTEN. We administered 1e9 transducing units (TU)/Kg (n=2) or 3e9 TU/Kg (n=3) dose for LV.coFVIII.XTEN or 3e9 TU/Kg (n=2) or 6e9 TU/Kg (n=3) dose for LV.coFVIII. A corticosteroid immune-suppression regimen was applied from day -1/3 to day +7/9, since human FVIII is known to be highly immunogenic in NHP. Administration of LV via peripheral vein was well tolerated with no significant changes in body temperature. Selflimiting leukopenia and limited serum aspartate aminotransferases (AST) elevation were observed. Therapeutically relevant FVIII amounts were observed in all treated animals, with the target 60-100% of normal human FVIII activity achieved at 3e9 TU/Kg dose for the LV.coFVIII. XTEN treatment group. We monitored both anti-FVIII antibody (Abs) formation, acute cytokine response to LV administration and T cell responses in the blood and the spleen of treated NHP. Upon corticosteroids discontinuation, all NHP developed anti-FVIII Abs, but 4/5 LV.coFVIII.XTEN treated NHP maintained LV-positive hepatocytes in the liver at the end of the study and their splenocytes did not respond to *ex vivo* FVIII stimulation. On the contrary, only 1/5 LV.coFVIII treated NHP maintained LV-positive hepatocytes in the liver and all of them showed splenocyte activation after FVIII stimulation *ex vivo*. Overall, our data show efficient and well tolerated gene transfer to the liver of NHP by LV, with an improved therapeutic index for the engineered FVIII.XTEN transgene, supporting further pre-clinical and potentially clinical development of this gene therapy strategy.

38. Towards Clinical Translation of Hematopoietic Cell Gene Editing for Treating Hyper-IgM Type 1

Valentina Vavassori*[1,2], Elisabetta Mercuri*[1], Genni Marcovecchio[1], Maria Carmina Castiello[1,3], Daniele Canarutto[1,2], Claudia Asperti[1], Aurelien Jacob[1], Luisa Albano[1], Elena Fontana[3,4], Eugenio Scanziani[5], Marina Radrizzani[1], Anna Villa[1,3], Pietro Genovese#[1], Luigi Naldini#[1]

[1]SR-TIGET, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]CNR, Milan, Italy,[4]Humanitas, Milan, Italy,[5]Fondazione Unimi, Milan, Italy

HIGM1 is caused by mutations of CD40L, whose absence in CD4 T cells impairs signaling for B cell activation and Ig class-switching. Since unregulated CD40L expression leads to lymphoproliferation/ lymphoma, gene correction must preserve its physiological regulation. Gene editing of either autologous T cells or hematopoietic stem cells (HSC) held promise for treating HIGM1. We developed a “one size fits all” editing strategy to insert a 5’-truncated corrective CD40L cDNA in the first intron of the native human gene, effectively making expression conditional to targeted insertion in the intended locus. By exploiting a protocol that preserves T stem memory cells (TSCM), we reproducibly obtained ~40% of editing efficiency in healthy donor and patients derived T cells, restoring regulated, although partial, CD40L surface expression that was sufficient to restore helper function on B cell co-cultures. To select, track and potentially deplete edited T cells, we coupled the corrective cDNA with a clinically compatible selector gene and confirmed that enriched T cells preserved their engraftment capacity in NSG mice. Unexpectedly, the presence of an IRES-linked downstream coding frame counteracted the shorter half-life of transcript from the edited locus, allowing replenishment of intracellular stores and surface translocation of physiological amounts of CD40L upon activation. We also tailored the *CD40L* editing strategy to human HSC, reaching up to 15-30% editing in HSC long term engrafting NSG mice, depending on the HSC source. We then modelled the therapeutic potential of both T cell and HSC gene therapy by infusing increasing proportions of WT murine cells, as surrogates of edited cells, in HIGM1 mice. Administration of functional T cells at clinically relevant doses in HIGM1 mice, preconditioned or not with different lymphodepleting regimens, achieved long term stable T cell engraftment and partial rescue of antigen specific IgG response and germinal center formation in splenic follicles after vaccination with a thymus dependent antigen.

Remarkably, infusion of T cells from mice pre-exposed to the antigen, mimicking treatment of chronically infected patients, was

effective even in absence of conditioning and protected the mice from a disease relevant infection induced by the opportunistic pathogen Pneumocystis murina. Transplantation of functional T cells admixed with HIGM1 T cells resulted in lower vaccination response, indicating competition between WT and HIGM1 cells and implying that increasing the fraction of corrected cells in the graft by selection would improve immune reconstitution. Concerning HSC gene therapy, transplanting 25% WT cells along with HIGM1 ones in HIGM1 mice - mirroring the editing efficiencies achieved in human HSC - rescued antigen specific IgG response and established protection from pathogen comparably to T cell therapy. These findings suggest that autologous edited T cells can provide immediate and substantial benefits to HIGM1 patients and position T cell as competitive strategy to HSC gene therapy, because of more straightforward translation, lower safety challenges and potentially comparable clinical benefits. We thus embarked in assessing GMP compliant reagents and protocols for T cell activation, culture and editing and developed a scalable manufacturing process. Optimization of clinical grade culture conditions allowed further increasing editing efficiency, total cellular yield and maintenance of TSCM thus paving the way to the design of a clinical trial.

39. A Phase 1/2 Study of Lentiviral-Mediated Ex-Vivo Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I): Interim Results

Donald B. Kohn[1], Gayatri Rao[2], Elena Almarza[3], Dayna Terrazas[1], Eileen Nicoletti[4], Augustine Fernandes[1],
Caroline Kuo[1], Satiro De Oliviera[1], Theodore Moore[1], Ken Law[4], Brian Beard[4], Julian Sevilla[5], Cristina MesaNunez[6], Claire Booth[7], Adrian Thrasher[7], Juan Bueren[6],
Jonathan Schwartz[4]

[1]UCLA, Los Angeles, CA,[2]Rocket Pharmaceuticals, Inc., Cranbury, NJ,[3]Rocket Pharmaceuticals, Inc, New York, NJ,[4]Rocket Pharmaceuticals, Inc, Cranbury, NJ,[5]Pediatric Oncology Hematology and Stem Cell Transplant Department, FIB Hospital Infantil Universitario Niño Jesús, and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[6]Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación
Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[7]Infection, Immunity, & Inflammation Department, UCL Great Ormond Street (GOS) Institute of Child Health, London, United Kingdom

Introduction: LAD-I is a rare disorder of leukocyte (primarily neutrophil) adhesion resulting from *ITGB2* gene mutations encoding for the β2-integrin component, CD18. Severe LAD-I (i.e., CD18 expression on <2% of PMNs) is characterized by severe infections, impaired wound healing, and childhood mortality. Although allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative, utilization and efficacy are limited by donor availability and risk of graft-versus-host disease (GVHD). RP-L201-0318 (NCT03812263) is a phase 1/2 open-label clinical trial evaluating the safety and efficacy of RP-L201, consisting of autologous CD34+ cells transduced with a lentiviral vector (LV) carrying the *ITGB2* gene encoding for CD18 (Chim-CD18-WPRE) in severe LAD-I. **Methods:** Pediatric patients

≥ 3 months old with severe LAD-I are eligible. Peripheral blood (PB) HSCs are collected via apheresis after mobilization with granulocytecolony stimulating factor (G-CSF) and Plerixafor and transduced with Chim-CD18-WPRE LV. Myeloablative busulfan conditioning is followed by RP-L201 infusion. Patients are followed for safety and efficacy (i.e., survival to age 2 and at least 1-year post-infusion, increase in PMN CD18 expression to at least 10%, PB vector copy number (VCN), decrease in infections/hospitalizations, and resolution of skin or periodontal abnormalities). **Results:** Four patients (ages 7 months to 9 years) have been treated with RP-L201 with follow-up ranging from 6 weeks to 12 months. RP-L201 cell doses ranged from $2.8x10^6$ to $6.5x10^6$ CD34+ cells/kg with VCNs from 1.83 to 3.8 copies/cell (liquid culture). No serious treatment-emergent adverse events were reported. Neutrophil engraftment was observed in ≤ 5 weeks. PB PMN CD18 expression in Patient 1 12-months post-treatment was 40% (sustained from 47% at 6-months, vs. < 1% at baseline), with PB VCN of 1.2. Skin lesions present at baseline resolved with no new lesions reported. Patient 2 PB PMN CD18 expression 6-months post-treatment was 23% with PB VCN at 0.75. Patients 3 and 4 PB PMN CD18 expression were 74% at 3-months and 59% at 6-weeks post-treatment, respectively. No new infections have been reported in patients post-infusion. **Conclusion:** These results demonstrate that RP-L201 leads to durable neutrophil CD18 expression and improved clinical course. Additional patient treatment is planned for early 2021.

40. Autologous Ex Vivo Lentiviral Gene Therapy for the Treatment of ADA-SCID

Claire Booth[1], Don B. Kohn[2], Kit L. Shaw[2], Jinhua Xu-

[1] SR-TIGET, Ospedale San Raffaele, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]Milano-Bicocca University, Monza, Italy

Hematopoietic stem/progenitor cells (HSPCs) gene editing based on homology directed repair (HDR) prospectively allows the treatment of human hematological diseases by *in situ* gene correction while maintaining physiologic regulation. However, in most hematological diseases, gene corrected HSPC would not gain selective advantage over the unedited counterpart; therefore, current HDR editing efficiencies might be insufficient to establish robust therapeutic benefit. Selection of HDR-edited HSPCs, followed or not by ex vivo expansion, would allow administering higher numbers and/or proportions of corrected cells, improving efficacy of HSC therapy while lowering the conditioning requirement and the toxicity of the procedure. Here, we developed selection strategies that couple gene correction and transient Selector expression by Means of Artificial Transcription activators (SMArT). In one design, we fused the

Bayford[3], Elizabeth Garabedian[4], Valentina Trevisan[1], Denise A. Carbonaro-Sarracino[5], Kajal Soni[1], Dayna Terrazas[2], Katie Snell[5], Diego Leon-Rico[1], Karen Buckland[1], Kimberly Gilmour[1], Satiro De Oliveira[2], Christine Rivat[5], Natalia Izotova[1], Stuart Adams[1], Hilory Ricketts[1], Alejandra Davila[2], Chilenwa Uzowuru[1],
Beatriz Campo Fernandez[2], Roger P. Hollis[2], Maritess Coronel[2], Ruixue Zhang[2], Serena Arduini[5], Frances Lynn[5], Mahesh Kudari[5], Andrea Spezzi[5], Marco Zhan[6], Rene Heimke[6], Ivan Labik[6], Kenneth Cornetta[7], Robert Sokolic[4], Michael Hershfield[8], Manfred Schmidt[6], Fabio Candotti[9], Harry L. Malech[10], Adrian J. Thrasher[1], H.
Bobby Gaspar[5]
[1]UCL GOSH Institute of Child Health, London, United Kingdom,[2]University of California, Los Angeles, Los Angeles, CA,[3]Great Ormond Street Hospital NHS Trust, London, United Kingdom,[4]National Human Genome Research Institute, Bethesda, MD,[5]Orchard Therapeutics, London, United Kingdom,[6]GeneWerk, Heidelberg, Germany,[7]Indiana University School of Medicine, Indianapolis, IN,[8]Duke University, Durham, NC,[9]Lausanne University Hospital, Lausanne, Switzerland,[10]National Institute of Allergy and Infectious Diseases, Bethesda, MD

Introduction: Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) is a rare and life-threatening disorder caused by *ADA* gene mutations leading to compromised immune function. Current treatment guidelines suggest enzyme replacement therapy prior to definitive treatment with hematopoietic stem cell transplantation or gene therapy. An investigational gene therapy (GT) consisting of autologous $CD34^+$ hematopoietic stem cell progenitors (HSPCs) transduced *ex vivo* using a self-inactivating lentiviral vector (LV) encoding the human *ADA* cDNA sequence under the control of a shortened human elongation factor 1α gene promoter (EFS-ADA LV) was studied in trials in the US and EU. Methods: Fifty patients with ADA-SCID (30 in the US and 20 in the EU) were treated with lentiviral gene therapy following nonmyeloablative busulfan conditioning. An analysis was conducted integrating two US studies (using fresh and cryopreserved formulations of OTL-101) at 24-months' follow-up alongside an EU study (fresh formulation) with 36-months' follow-up. Results: Overall survival was 100% for all analyses up to 24 and 36 months. Event-free survival (in the absence of enzyme replacement therapy reinstitution or rescue allogeneic hematopoietic stem cell transplant) was 96.7% (US studies) and 100% (EU patients) at 12 months, 96.7% and 95%, respectively at 24 months, and 95% (EU patients) at 36 months. Engraftment of genetically modified HSPCs persisted in 29/30 US patients and 19/20 EU patients up to last follow-up. Patients exhibited sustained metabolic detoxification and normalized ADA activity levels. Immune reconstitution was robust with T cell counts reaching or approaching normal ranges at last follow up, including increased naïve T cell numbers. 89.7% of US patients and 100% of EU patients discontinuing immunoglobulin replacement therapy by 24 and 36 months, respectively demonstrating improved B cell function. Most adverse events were mild. No evidence of monoclonal expansion, leukoproliferative complications, or emergence of replication-competent lentivirus was noted and no events of autoimmunity or graft-versus-host disease occurred. Conclusions: Treatment of ADA-SCID with *ex vivo* lentiviral HSPC gene therapy resulted in high rates of overall and event-free survival, with sustained *ADA* gene expression, metabolic correction, and functional immune reconstitution.

41. Efficient Ex-Vivo Selection of Gene Edited Human Hematopoietic Stem/Progenitor Cells

Martina Fiumara*[1,2], Samuele Ferrari*[1], Elisabetta Mercuri[1], Aurelien Jacob[1,3], Luisa Albano[1], Angelo Lombardo[1,2], Pietro Genovese#[1], Luigi Naldini#[1,2]

self-cleaving 2A peptide. Since transcription of the edited gene is often low in HSPCs and might not suffice for detectable selector expression, an ArT directed against the targeted gene promoter was co-delivered by mRNA with the editing machinery. Upon editing therapeutically relevant genes in human HSPCs, we achieved transient selector overexpression in all HSPC subpopulations, allowing enrichment of HDR-edited cells up to 90% in the sorted fraction. To avoid selector coexpression in the HSPC progeny physiologically expressing the edited gene, which in some setting may cause toxicity and/or immunogenicity, we improved the SMArT design by introducing in the HDR-template an independent selector cassette regulated by a minimal promoter (minP) that is nearly inactive at basal level. Transient delivery of an ArT binding on the genome flanking the minP but outside the homology region comprised in the HDR-template induced selector expression only in HDR-edited HSPCs. Transactivation, however, was weaker in the more primitive HSPC subpopulations, likely due to the distance between the Art binding site and the transcriptional start site (TSS). To overcome this limitation, we modified the SMArT HDR-template by incorporating multiple tetracycline operator (TetO) repeats close to the minP and transiently co-expressed a tetracyclineregulated TransActivator (tTA) by RNA. Multiplexed ArT binding in close proximity of the TSS allowed robust and transient selector expression in the most primitive HSPC subpopulations. We then tuned tTA activity by an initial pulse of doxycycline administration to suppress transactivation while non-integrated template is still in excess and reached up to 100% HDR editing in the selector-positive fraction. SMArT was portable to several disease relevant loci (*IL2RG*, *AAVS1* and *CD40LG*), compatible with different HSPCs sources and with clinically compliant selectors. On-going experiments are aimed to stringently evaluate the repopulation potential of SMArT-selected HSPCs. Overall, we anticipate that enrichment of HDR-edited HSPCs by SMArT would allow to broaden clinical applicability and tolerability of HSC gene editing.

42. Targeted Genome Editing of Hematopoietic Stem Cells for Treating Recombination Activating Gene 1 (RAG1)

selector (e.g. GFP, ΔNGFR) open-reading frame with the corrective coding sequence of the targeted gene by a

Immunodeficiency

Maria Carmina Castiello[1,2], Nicolò Sacchetti[1], Elena Draghici[1], Samuele Ferrari[1], Valentina Vavassori[1], Chiara Brandas[1], Denise Minuta[1], Martina Di Verniere[1], Aurelien Jacob[1], Enrica Calzoni[1,3], Marita Bosticardo[3], Luigi Daniele Notarangelo[3], Luigi Naldini[1], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche (CNR), Milano, Italy,[3]Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda, MD

Recombination Activating Genes (*RAG*) are tightly regulated molecules during T and B cell differentiation. Mutations in *RAG* genes result in a broad spectrum of immunological disorders including T-B- SCID and leaky forms. Curative treatment is hematopoietic stem cell transplantation but donor availability is still limited and unsatisfactory outcomes have been described with partially HLA-matched donors. Gene replacement therapy have been studied in pre-clinical models, however safety concerns are related to unregulated *RAG1* expression.

Thus, we developed a specific genome editing (GE) strategy, based on the delivery of engineered nucleases and DNA template, to correct *RAG1* mutations and restore gene expression and function. We firstly assessed the minimal therapeutic dose of functional cells by competitive transplant experiments into *Rag1*$^{-/-}$ mice, which showed that low proportion of wild-type or *Rag1*$^{+/-}$ lineage negative bone marrow cells can correct immune defects. We exploited the CRISPR/Cas9 platform combined with the delivery of an AAV6 donor carrying the corrected codon sequence downstream splice acceptor site sequence to use the endogenous *RAG1* promoter. A panel of 12 guides was screened and we selected the best performing one in terms of cutting efficiency assessed by T7 surveyor assay. The selected guide delivered with the corrective AAV6 donor resulted in restoration of RAG1 activity assessed by an in vitro LV-based recombination assay in Rag-1 KO cells. We apply our GE strategy to human CD34+ derived from cord blood or mobilized peripheral blood (MPB) of healthy donors (HD) and we obtained high editing efficiency even in the most primitive hematopoietic stem and progenitor cells (HSPC), assessed as homology directed repair (HDR) efficiency by ddPCR. We exploited an organoid platform to assess the T cell differentiation potential of edited HSPC coupling the GE and the T cell differentiantion protocols optimized for our purposes. Human CD34+ cells edited in *RAG1* locus differentiated in CD3+ TCRαβ+ cells demonstrating that our strategy preserves the T cell differentiation potential. Next, we edited CD34+ cells derived from MPB of HD and two RAG1 patients carrying hypomorpic *RAG1* mutations resulting in a reduced recombination activity and presenting with a combined immunodeficiency with granuloma formation and autoimmunity. Edited cells were transplanted into NSG mice to assess the engraftment capability and kept in culture for phenotypical and molecular analyses. The HPSC phenotype and HDR efficiency (40%) were similar between HD and RAG1-derived edited cells *in vitro.* Importantly, GE did not impact the engraftment and multilineage differentiation of edited HSPC in NSG mice. Moreover, we observed a peripheral selective advantage of edited cells in NSG mice transplanted with edited CD34+ cells derived from RAG1 patients.Overall, our findings suggest that we set up an efficient and promising GE platform for the correction of RAG1 deficiency, that would potentially benefit treatment of RAG1 patients who lack matched donors.

Musculo-skeletal Diseases

43. The Long-Term Efficiency of the scAAV. U7.ACCA Vector in Inducing Dystrophin Expression in Adult Dup2 Mice

Liubov V. Gushchina, Adrienne J. Bradley, Kelly M. Grounds, Aisha Suhaiba, Emma Frair, Calli Bellinger, Tabatha Simmons, Natalie Rohan, Nicolas Wein, Kevin M. Flanigan

Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH

Duplications in exon 2 of the *DMD* gene, encoding the dystrophin protein, account for around 10% of all duplication mutations associated with the X-linked Duchenne muscular dystrophy (DMD). As part of

the preclinical development of a U7snRNA vector currently in a clinical trial, our group has previously evaluated the therapeutic efficacy, absence of off-target splicing effects in AAV9.U7snRNA mediated skipping of exon 2 in a murine Dmd model, and lack of toxicity in non-human primates. Here we report long-term dystrophin expression data following treatment of 3-month-old Dup2 mice with the scAAV. U7.ACCA vector, which contains four copies of U7snRNA targeted to the exon 2 splice acceptor and splice donor sites. Dup2 males received a single intravenous infusion of 3E13 vg/kg, which is the minimal efficacious dose (MED) dose in ongoing first-in-human clinical trial (ClinicalTrials.gov NCT04240314). The age matched Dup2 treated with diluent and C57Bl/6 mice were used as controls. All animals (n=9-11) were sacrificed 18-month post vector administration. The RT-PCR results showed that a single scAAV9.U7.ACCA vector injection resulted in a significant exon 2 skipping in tibialis anterior (TA), diaphragm (Dia) and heart tissues, showing an average of 46%, 32% and 73% total therapeutic transcripts, respectively. To determine the degree of functional rescue, we performed in situ physiology studies on TA and in vitro on Dia muscles. Treated with diluent, both Dia and TA muscles from 21-month-old Dup2 mice exhibited a functional deficit with a significant (45-61%) reduction in specific force output compared with C57Bl/6 (Bl6) mice. The significant force drop was also

observed in diluent treated Dup2 mice compared with Bl6 mice following a rigorous fatigue protocol. The single scAAV9.U7.ACCA injection resulted in a dramatic improvement in specific force output, which increased up to 64-76% in Dia and TA muscles, respectively, and better protection of the TA muscle from repeated fatigue, which improved up to 73%. Overall, these data support our previous findings showing that scAAV9. U7.ACCA provides long-term protection by restoring the disrupted dystrophin reading frame in straight muscles from Dup2 mice and functional recovery of TA and Dia muscles 18-month post vector administration.

44. Towards an Off-the-Shelf Cell Therapy for Bone Healing: Use of an Immortalized, Genetically Modified Cell Line as a Proof of Concept

Rodolfo E. De la Vega[1,2], Michael J. Coenen[1], Gresin Hawse[1], Joseph A. Panos[1], Christopher H. Evans[1]

[1] Rehabilitation Medicine Center, Mayo Clinic, Rochester, MN,[2]cBITE, MERLN Institute - Maastricht Unversity, Maastricht, Netherlands

Introduction: Bone morphogenetic protein-2 (BMP-2) is the most potent clinically available osteogenic cytokine. Yet, after almost two decades in the market, it has limited clinically approved use conditions, requires supraphysiological doses, and side effects are a concern. Gene transfer has been suggested as a means to improve and prolong delivery while lowering side effects. *Ex vivo* gene transfer using autologous cells has been studied in this regard but is limited by the high cost associated with harvesting, tissue culture expansion, transduction, and characterization under GMP conditions prior to re-implantation. This project explores an alternative approach by generating and evaluating a stably-transduced, BMP-2 expressing, immortalized cell line to serve as a proof of concept for an eventual off-the-shelf, allogeneic cell therapy product for bone healing in humans. METHODS: We used HEK293 cells because of their high proliferation rate, ease of maintenance and transduction. Cells were transduced with a lentivirus coding for the human BMP-2 gene driven by the CMV promoter. Clones were selected by puromycin, expanded, BMP-2 production characterized by ELISA and cells frozen at either $5x10^5$ (low), $1x10^6$ (medium) or $3x10^6$ (high) cells/vial. Individual vials were thawed and cells encapsulated in fibrin just prior to surgical implantation in a rat, 5-mm, femoral, critical size bone defect model. The high dose of 11 µg BMP-2 bridges the defect, while empty defects fail to bridge, acting as positive and negative controls, respectively (n=10/group). FK506 was used to prevent xenograft rejection. Defect bridging was monitored via weekly radiographs until euthanasia at 12 weeks. A subset of animals receiving $1x10^6$ cells (n=5) was euthanized at 4 weeks for gDNA extraction from the defect site to determine the presence of implanted cells. RESULTS: Two clones were selected and expanded. Clone CL1K showed significantly higher BMP-2 production per cell than CL20K (Fig. 1A). CL1K was expanded and BMP-2 production was confirmed up to passage 50 (Fig. 1B). After 12 weeks (Fig. 1C), defect bridging seemed to be correlated to the number of implanted cells, with 20%, 60% and 70% of animals bridging in the low, medium and high groups, respectively. qPCR was able to detect gDNA from the implanted cell line in 3 out of 5 animal samples, with one of these animals presenting with solid mass at the femoral defect site. DISCUSSION: Although 293 cells cannot differentiate into osteoblasts, the BMP-2 expressing CL1K cells were able to bridge large bone defects. It is widely assumed that *ex vivo* gene delivery for bone healing must use osteoprogenitor cells as vehicles. However, these data show otherwise and suggest a novel approach to bone healing. We know of no other reports where a cell unable to differentiate into osteoblasts has been used to induce bone healing in a bone defect model. 293 cells are not an ideal candidate for *ex vivo* therapies because of the risk of tumorigenesis, but this experiment serves as a proof of concept for the prospect of an allogeneic off-the-shelf cell line for bone healing. CONCLUSIONS: These novel findings provide a basis for the development of a convenient and effective *ex vivo* gene therapy product for bone healing. Future work is needed to optimize the conditions needed for this approach to be successful for clinical translation.

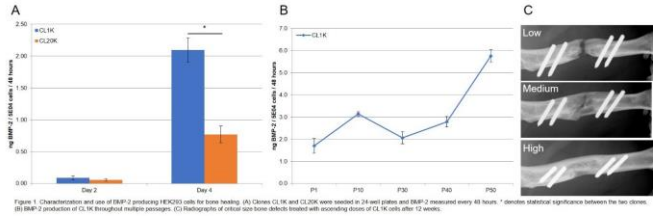

Figure 1. Characterization and use of BMP-2 producing HEK293 cells for bone healing. (A) Clones CL1K and CL20K were seeded in 24-well plates and BMP-2 measured every 48 hours. * denotes statistical significance between the two clones. (B) BMP-2 production of CL1K throughout multiple passages. (C) Radiographs of critical-size bone defects treated with ascending doses of CL1K cells after 12 weeks.

45. Correction of Clcn1 Mis-Splicing Reverses Muscle Fiber Type Transition in Mice with Myotonic Dystrophy

Ningyan Hu, Thurman Wheeler

Massachusetts General Hospital, Boston, MA

Background: DM1 is caused by an expanded CTG repeat in the DMPK gene. Characteristics of this multisystem disorder include myotonia (delayed muscle relaxation), progressive weakness, muscle wasting, and cardiac conduction defects. Clinical features of DM1 arise from expression of DMPK transcripts that contain expanded CUG repeats (CUG)exp that accumulate in nuclear inclusions of skeletal muscle and other affected tissues. This pathogenic RNA readily binds proteins in the muscleblind-like (MBNL) family that are required for normal regulation of alternative splicing, resulting in loss of MBNL protein function. In the HSALR transgenic and Mbnl1 knockout mouse models of DM1, alternative splicing patterns in muscle tissue are very similar. A clinical manifestation of Mbnl1 loss of function includes myotonia, a delayed muscle relaxation caused by mis-splicing of muscle chloride channel Clcn1. In human DM1 muscle, oxidative muscle fibers are upregulated and preferentially atrophic as compared to glycolytic fibers. **Methods**: We crossed HSALR transgenic mice with Mbnl1 knockout mice to create a double homozygous mouse model of DM1. Tibialis anterior muscles were injected with an antisense morpholino oligo that target Clcn1 exon 7a for skipping. The contralateral muscle were injected with the 5' - 3' invert of the active drug. We measured Clcn1 splicing by RT-PCR, transgene expression

by droplet digital PCR, and myosin fiber type by immunolabeling and ddPCR.
Results: In muscles receiving the active treatment, Clcn1 splicing was corrected by 16 days after injection. Type 2B glycolytic muscle fibers were nearly absent in untreated double mutant mice and 50% of the overall total in treated mice. Expression of ACTA1-CUG transcripts and the muscle regeneration marker embryonic myosin (Myh3) was reduced. **Conclusions**: Chronic severe myotonia results in muscle fiber type transition from glycolytic to oxidative in models of DM1. Reversal of Clcn1 mis-splicing is sufficient to rescue muscle fiber type patterns and reduce muscle fiber damage. **Grant support**: Muscular Dystrophy Association.

46. Characterization of Acute Toxicity after High-Dose Systemic Adeno-Associated Virus in Nonhuman Primates, Including Impact of Vector Characteristics

Juliette Hordeaux, Chunjuan Song, Erik Wielechowski, Ali Ramezani, Cecilia Dyer, Elizabeth L. Buza, Jessica Chichester, Peter Bell, James M. Wilson
Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Dose-limiting toxicities have occurred following intravenous administration of high doses of adeno-associated virus (AAV) to target the musculoskeletal or central nervous systems. Acute elevations in liver enzymes and/or reductions in platelets have been observed in most high-dose AAV clinical trials. Although infrequent, severe toxicities have included anemia, renal failure, complement activation, and, in the worst cases, fatal hepatobiliary disease. We previously reported the development of acute thrombocytopenia and transaminitis in most nonhuman primates (NHPs) within days of of receiving high doses of AAV (in the 1x1014 GC/kg range). Some animals recover while some develop a lethal syndrome of coagulopathy, liver failure, hemorrhage, and shock. The acute initial presentation of thrombocytopenia and transaminase elevation is common to NHPs and humans and has not been observed in other species in our experience. Although the progression of the initial acute toxicity may differ in human patients with pre-existing conditions compared to healthy animals, we propose that NHPs can be used as a model to better characterize the acute toxicity of high doses of AAV to reduce risk in clinical trials. We conducted studies aimed at investigating the effect of capsids, prophylactic steroids, and vector purification methods on the

incidence and severity of acute toxicity following high-dose AAV systemic administration. We administered ten rhesus macaques (16 months to 4.6 years old with anti-capsid neutralizing titers < 5 and weighing 3-7 kg) with AAV9 or an engineered capsid derived from AAV9 (AAV-PHP.eB) encoding green fluorescent protein at 1 or 2 x 1014 GC/kg. We then monitored for acute toxicity for 2 weeks post-administration. All vectors were produced from transient transfection of adherent HEK293 cells and either purified using affinity column chromatography or an iodixanol gradient. Both purification methods yielded more than 70% of full capsids. We observed acute toxicity in the majority of animals, regardless of the purification method, characterized by thrombocytopenia, liver enzyme elevation with or without hyperbilirubinemia, and increased coagulation times on day 3 post-administration. Most animals recovered from the initial toxicity except for two AAV-PHP.eB-dosed animals that had to be humanely euthanized due to severe coagulopathy. Prophylactic steroids appeared to help with the recovery from the initial toxicity but did not prevent it from occurring. The toxicity was dose dependent, with AAV9 capsid having a narrow safety margin: 1 x 1014 GC/kg was well tolerated whereas a 2-fold increase led to thrombocytopenia in the majority of animals. Importantly, we observed complement activation concurrent with the thrombocytopenia on day 3 with activation of the alternate pathway and elevation of complement Bb fragment and SC5b-9 membrane attack complex, whereas the classical pathway did not appear to be activated (unchanged C4 and C4a levels). Accordingly, there were no detectable IgM antibodies to the capsid on day 3, suggesting that immune complexes were not the cause of toxicity at 3 days post-administration in NHPs. Collectively, our data suggest that the acute toxicity that occurs after high-dose AAV in NHPs causes thrombocytopenia and liver injury on day 3 post-administration and resolves by day 7 in the majority of animals. This toxicity is capsid dependent, dose dependent, unrelated to a vector purification method, and involves transient activation of the alternate complement pathway.

47. Long-Term Hematopoietic Stem Cell Lentiviral Gene Therapy Corrects Neuromuscular Manifestations in Preclinical Study of Pompe Mice

Niek P. van Til[1,2], Yildirim Dogan[1], Cecilia Barese[1], Zeenath Unnisa[1], Swaroopa Guda[1], Rena Schindler[1], John Yoon[1], Mary Jacobs[1], Abhishek Chiyyeadu[1], Daniella Pizzurro[1], Bianling Liu[1], Mirjam Trame[1], Tim Maiwald[1], Christine Oborski[1], Axel Schambach[3], Claudia Harper[1], Richard Pfeifer[1], Chris Mason[1,4]
[1]AVROBIO, Inc, Cambridge, MA,[2]Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit and Amsterdam Neuroscience, Amsterdam, Netherlands,[3]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,[4]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

Pompe disease is an inherited disorder caused by acid alpha-glucosidase (GAA) deficiency, leading to lysosomal glycogen accumulation in the heart, skeletal muscles, and the central nervous system (CNS). Pompe disease presents with generalized muscle weakness, and, if untreated, the most severely affected patients typically succumb

early in life due to cardiorespiratory failure. The standard of care is enzyme replacement therapy, which requires lifelong treatment, but does not prevent disease progression. Hence, there is a clear unmet medical need to develop treatment options for Pompe disease patients that could provide a lifelong, life-altering benefit. We used

a lentiviral vector driven approach to overexpress a chimeric human *GAA* transgene with a glycosylation independent lysosomal targeting (GILT) tag in the hematopoietic system of a Pompe disease mouse model. A novel therapeutic lentiviral vector carrying a clinically proven promoter was selected and tested for long-term efficacy in Pompe mice. Supraphysiological GAA enzyme activity levels were achieved in hematopoietic cells and plasma following transplantation with genetically modified hematopoietic stem and progenitor cells (HSPCs) in pre-conditioned Pompe mice. Urinary hexose tetrasaccharides (Hex4), a breakdown product of glycogen, were reduced in treated Pompe mice. GAA enzyme activity was detected in the murine heart, skeletal muscles, spinal cord, and at lower levels in the brain at eight months after transplantation. Glycogen clearance was complete in the murine heart, diaphragm, brain, spinal cord, and in most of the skeletal muscles tested. Consequently, pathological heart remodeling was reversed. Locomotor function was improved. Average vector copy numbers in leukocytes were below five in all the experimental groups. Importantly, administering the lowest dose median VCN ~2 provided robust glycogen reduction indistinguishable from higher doses (median VCN >3) administered. In addition, glucose levels remained stable during the study and the hematopoietic reconstitution of transplanted gene-modified HSPCs was similar to controls. In summary, our candidate vector was shown, in mouse models, to be a potentially effective therapeutic approach for long-term alleviation of cardiomyopathy, muscle weakness and CNS pathology in Pompe disease. We believe this approach could translate into a clinical application of single-dose therapy for Pompe disease patients.

48. Downregulation of the Genetic Modifier *PITPNA* as Means of Therapy in Duchenne Muscular Dystrophy

Matthias R. Lambert, Yuanfan/Tracy Zhang, Janelle M. Spinazzola, Jeffrey J. Widrick, James R. Conner, Louis M. Kunkel

Genetics and Genomics, Boston Children's Hospital, Boston, MA

Duchenne muscular dystrophy (DMD) is a severe genetic disorder caused by mutations in the *DMD* gene. Absence of dystrophin protein leads to progressive degradation of muscle function and leads to premature death. Although there are several promising strategies under investigation to restore dystrophin protein expression, there is currently no cure for DMD, and the development of dystrophinindependent therapies is essential. The recent advances in wholegenome/exome sequencing and the use of large-scale databases have enabled the identification of several genetic modifiers that influence clinical presentation and represent an unexplored territory for therapy. In our lab, we identified *PITPNA* as a new genetic modifier of DMD in two exceptional mildly affected Golden Retriever Muscular Dystrophy (GRMD) dogs. Downregulation of *PITPNA* allowed dystrophindeficient dogs to retain functional muscle and normal lifespan. This was confirmed in our dystrophin-deficient zebrafish in which *pitpna* downregulation by morpholino antisense nucleotide resulted in improved muscle structure and function. In the present study, we explored different strategies that both aimed to downregulate *PITPNA* and prevent the muscle pathology. In dystrophin-deficient zebrafish, we developed a straightforward phenotypic drug screening assay that would be inconceivable in mouse model systems. We identified that phosphodiesterase 10A (PDE10A) inhibitors improved muscle integrity and reduced *pitpna* expression. The PDE10A pathway was confirmed with the use of *pde10a* morpholino and we combined different functional assays that showed improvement in locomotion, muscle, and vascular function as well as long-term survival in *dmd* zebrafish. In dystrophin-deficient mice, we are currently evaluating the potential benefit of *Pitpna* downregulation on muscle pathology based on two different strategies: (i) the use of PDE10A inhibitors and (ii) the use of Pitpna shRNA-AAV. Despite recent advances in dystrophin replacement strategies, there is still precedence to pursue pharmacological therapies targeting genetic modifiers that can complement dystrophin-based therapies and are independent of patients' genetic mutations. In different animal models of DMD, downregulation of the genetic modifier *PITPNA* presents several reasons for there to be further studied of it as a potential DMD therapeutic via two strategies that may benefit patients.

49. Non-Genotoxic Conditioning to Increase Gene Therapy Safety in a Rare Bone Disease

Valentina Capo[1,2], Sara Penna[1,3], Ludovica Santi[1], Andrea Cappelleri[4,5], Stefano Mantero[2,6], Elena Fontana[2,6], Eugenio Scanziani[4,5], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific

Institute, Milan, Italy,[2]Milan Unit, CNR-IRGB, Milan, Italy,[3]Dimet, University of Milano-Bicocca, Monza, Italy,[4]Department of Veterinary Medicine, University of Milan, Lodi, Italy,[5]Mouse and Animal Pathology Lab (MAPLab), Fondazione UniMi, University of Milan, Milan, Italy,[6]Humanitas Clinical and Research Center

- IRCCS, Rozzano, Italy

Autosomal recessive osteopetrosis (ARO) is a rare genetic disease, affecting osteoclast differentiation or function. Most ARO patients present mutations in *TCIRG1* gene, encoding the a3 subunit of V-ATPase proton pump, necessary for the acidification of bone resorption lacunae by osteoclasts. Symptoms include dense and brittle bones, limited bone marrow cavity, anaemia and progressive nerve compression, leading to death in the first decade of life. To date, hematopoietic stem cell transplantation (HSCT) is the only therapeutic option, but it is limited by availability of HLA-matched donors, toxicity of conditioning regimens and significant morbidity. We and others proposed gene therapy (GT) as an alternative strategy to overcome donor-related issues. However, the burden of conventional myeloablative conditioning on patients remains a strong unmet clinical need. Thus, we evaluated the use of a novel non-genotoxic conditioning in GT setting of *TCIRG1*-dependent ARO. In particular, we tested antibody-drug conjugates (ADCs), formed by anti-cKit or anti-CD45 antibody conjugated to the saporin toxin, able to make space in bone marrow (BM) and to promote hematopoietic stem and progenitor cell (HSPC) engraftment without off-target toxicity in adult mice. Since ARO symptoms occur very early in life, we evaluated the HSPC depleting potential of ADCs on WT newborn mice, administered via temporal vein at 1 day of life. We sacrificed mice 2 days after treatment and observed partial HSPC depletion in BM, spleen and peripheral blood. Notably, we did not

observe histological lesions or increase in apoptosis (evaluated with Caspase 3 immunohistochemistry) due to organ toxicity in kidney, brain and BM of ADC-treated mice. To evaluate the efficacy of non-genotoxic conditioning in favouring HSPC engraftment early in life, we performed mismatched HSCT in WT ADC-conditioned mice, and compared it to transplantation after sublethal total body irradiation or without conditioning. Twenty weeks after transplant, we observed low but persistent donor chimerism in ADC-treated mice compared to irradiated controls in peripheral blood, BM, spleen and thymus.As reported in literature, very low level of donor cells engraftment is sufficient to restore bone phenotype in osteopetrotic mice. We hypothesized that ADC conditioning could successfully guarantee bone phenotype amelioration and reduction of the conditioning toxicity in the osteopetrotic mouse model. This approach may be even more advantageous in the GT setting, in which autologous HSCT avoids the risk of graft rejection. We plan to apply ADC conditioning on osteopetrotic mice before the transplant of lentiviral vector GT Lin- cells, to test the efficacy of our strategy on this severe bone disease model. *Acknowledgements.* This project has received funding from the European Calcified Tissue Society.

Novel AAV Capsids for Brain, Eye and Muscle Tissues

50. Endothelial-Tropic AAVs for Genetic Access to Whole-Brain Vasculature in WildType Mouse Strains Following Non-Invasive Systemic Delivery

Xinhong Chen, Damien A. Wolfe, Sripriya Ravindra Kumar, Timothy F. Miles, Erin E. Sullivan, Acacia M. Hori, Xiaozhe Ding, Viviana Gradinaru

BBE, Caltech, Pasadena, CA

The neurovascular unit (NVU) is a vital yet understudied component of the nervous system. Malfunction of non-neuronal cell types within the NVU, including endothelial cells, can facilitate the progression of neurological disorders (Yu et al, Frontiers in Neuroscience, 2020), but limited options for cell-type specific transgene delivery hamper its study. Adeno-associated virus (AAV) vectors for gene delivery to the brain are commonly administered via intra-cranial injections, resulting in tissue damage and limited, uneven spatial coverage. Systemic AAV delivery provides a non-invasive, brainwide alternative for genetic access. Having engineered vectors that efficiently cross the bloodbrain-barrier (BBB) with broad tropism in rodents (e.g. AAV-PHP. eB), we turned our focus to engineering cell-type-specific vectors that could access vasculature without targeting other components of the NVU. Using M-CREATE directed evolution (Kumar et al, Nature Methods, 2020), we identified a family of endothelial-enriched capsid variants, including one named AAV-CAP.X1. Following intravenous (I.V.) injection, AAV-CAP.X1 targets vasculature with high cell-type specificity and efficiency throughout the body, including the brain. After injecting 3E11 vg total of AAV-CAP.X1 packaging CAG-GFP into adult C57BL/6J mice, 97% (+/- 0.8%) of the GFP+ area in the hippocampus

are CD31+ (demonstrating specificity), and 73% (+/- 9.1%) of the CD31+ area in the hippocampus is GFP+ (proving efficiency; note that an increased dosage of 1E12 vg per mouse resulted in even greater CD31+ labeling without losing specificity). As AAV-CAP.X1 vascular infectivity in the periphery may complicate applications that focus on brain-specific endothelial transduction, we introduced point mutations on the AAV-CAP.X1 capsid and incorporated microRNA target sites into the cargo genome that successfully de-target AAV-CAP.X1 from the liver without impairing brain transduction. AAV-CAP.X1 can be used across multiple genetically diverse mouse strains, with efficient labeling of both capillaries and arteries in the brains of C57BL/6J, FVB/NJ, CBA/J, and BALB/cJ mice following I.V. administration. We also observed a significant increase in transduction compared to its parent capsid AAV9 on multiple human-derived cell lines *in vitro*. In its brain-targeted form, AAV-CAP.X1 could be paired with pre-clinical therapeutic cargo both to probe vascular contributions to neurological disease and to inform intervention strategies. More broadly, gene delivery via endothelial-tropic AAV capsids could, in principle, be applied to study diverse pathologies that may benefit from vascular remodeling. Our evolving knowledge regarding vascular pathology in COVID-19 that could underlie generalized organ dysfunction demonstrates the timeliness and potential importance of such vectors.

51. RNA-Driven Evolution of AAV Capsid Libraries Identifies Variants with High Transduction Efficiency in Non-Human Primate Central Nervous System

Mathieu Nonnenmacher, Shaoyong Li, Wei Wang, Matthew A. Child, Amy Z. Ren, Katherine Tyson, Nilesh Pande, Xiaodong Lu, Jiangyu Li, Xiao-Qin Ren, Jianyu Shang, Michael Hefferan, Jay Hou, Omar Khwaja

Voyager Therapeutics, Cambridge, MA

Widespread transduction of the central nervous system (CNS) by viral or non-viral vectors still represents a considerable challenge in gene therapy. Local delivery of Adeno-Associated Virus (AAV) vectors to the CNS by intraparenchymal or intrathecal administration typically results in strong but heterogeneous transduction and is associated with potential risks related to invasive delivery. By contrast, intravascular delivery of engineered AAV vectors capable of crossing the bloodbrain barrier should allow a broader CNS transduction, thanks to the high density of the brain vascular network. We applied our previously described RNA-driven biopanning TRACER platform to perform directed evolution of an AAV9 capsid library in cynomolgus monkeys (*Macaca fascicularis*). Following two rounds of intravenous dosing and

neuron-specific library selection, a synthetic pool of variants was tested by multiplexed RNA enrichment analysis. This yielded a series of capsid variants with enhanced performance relative to AAV9. Five selected candidates were tested individually by low dose intravenous injection and their tropism for the CNS analyzed by measuring transgene RNA expression, viral DNA biodistribution and immunohistochemistry. All five variants were markedly improved over AAV9, with a subset showing 10-fold or more improvement of transduction in the brain. The highest performing variant displayed more than 1,000-fold higher RNA expression in the brain and 100-fold higher in the spinal cord. Immunohistochemical analysis indicated that this enhanced new

variant displays a predominant neuronal tropism and widespread transduction of multiple brain regions including the cortex, thalamus, putamen and brainstem. Transduction was strikingly high in deep cerebellar nuclei. We propose that this novel capsid variant has potential for use in multiple CNS indications.

52. Expanding the Utility of Intravitreal AAV via a Capsid Variant That Overcomes Neutralization by Anti-AAV2 NAbs in Human Vitreous

Siddhant S. Gupte[1], Sanford L. Boye[1], Wei Li[1], Sergei Zolotukhin[1], Paul Gamlin[2], Siva Iyer[3], Shannon Boye[1]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Alabama Birmingham, Birmingham, AL,[3]Ophthalmology, University of Florida, Gainesville, FL

Purpose: Adeno-associated virus (AAV) based gene therapies are approved for treating retinal disorders. While subretinal injection (SRI) leads to efficient gene transfer, intravitreal injection (IVI) is being pursued as a less invasive approach but is complicated by the presence of neutralizing antibodies (NAbs) against AAV in the vitreous. ~70% of the population has pre-existing anti-AAV2 NAbs, which can reduce therapeutic transgene expression to sub-therapeutic levels. As such, the presence of NAbs in the serum is currently used as an exclusion criterion for clinical trials utilizing IVI of AAV. We previously showed that 10% of human vitreous samples screened (31/301) contained high levels of AAV2 NAbs (>95% inhibition of transduction at 1:4 dilution). Here, we evaluated matched vitreous/serum samples (from the same individual) to determine the correlation between respective levels of AAV2 NAbs. We also continued to evaluate the ability of P2-V1, an AAV capsid variant identified through directed evolution and screening in non-human primate (NHP) for the ability to evade neutralization in these samples. Lastly, we characterized the transduction of P2-V1 in macaque following IVI at a clinically relevant dose. The ultimate goal is to identify gene therapy vectors capable of successfully treating the largest proportion of patients. **Approach:** Self-complementary smCBA-mCherry and CBA-GFP vectors were packaged in AAV2, P2V1 or AAV2.7m8 via plasmid transfection. Vectors used in the NHP experiment also underwent affinity column purification. Vitreous and blood (processed to serum) were collected from patients undergoing vitreoretinal surgeries. They were screened for AAV2 NAbs by infecting HEK293 cells with AAV2-mCherry at MOI of $5x10^3$ in the presence of vitreous or serum at 1:4 dilution followed by 4-fold dilutions ranging from 1:10 to 1:10,240. Expression was quantified via flow cytometry. Samples reducing AAV2 transduction >95% at 1:4 dilution were classified as inhibitory. The NAb50 titer (reciprocal dilution at which 50% inhibition of transduction occurs) for each sample was determined. AAV2, P2-V1 and AAV2.7m8 were similarly compared head-to-head in the presence of inhibitory vitreous samples. 1e11vg of AAV2(benchmark) or P2-V1 CBA-GFP vector was IVI into macaque. Transgene expression was recorded in life at 3 and 6 weeks post injection by fluorescent fundoscopy and by immunohistochemistry after sacrifice in retinal sections. **Results:** In head-to-head comparisons using inhibitory vitreous samples, P2-V1 had a 4-64 fold higher NAb50 value than AAV2 in 20 of 29 samples, and AAV2.7m8 in 16 of 22 samples. P2-V1 displayed higher transduction efficiency following IVI in NHP retina compared to AAV2. Of the 19 matched vitreous and serum samples tested so far, 9 serum and 4 vitreous samples inhibited AAV2. All 4 inhibitory vitreous samples had corresponding neutralizing serum, albeit with 4-64 fold higher NAb50. **Conclusions:** P2-V1 outperformed AAV2 and AAV2.7m8 in the presence of human vitreous samples containing anti-AAV2 NAbs and showed higher transduction than AAV2 in NHP retina. While more samples are being assessed, our results with matched serum and vitreous samples suggest that serum NAb levels are a more sensitive measure of a patient's NAb titer than vitreous. We are exploring whether blood/retinal barrier integrity is predictive of higher vitreous NAbs. The ability of P2-V1 to evade neutralization by the matched samples containing anti-AAV2 NAbs will also be studied. Use of P2-V1 may expand the number of patients that can participate in IVI-based clinical trials.

53. Breaking Thru the Human Blood Brain Barrier: Discovering AAV Vectors Targeting the Central Nervous System Using a Transwell Model

Ren Song[1], Katja Pekrun[1], Themasap A. Khan[2], Feijie Zhang[1], Sergiu Pasca[2], Mark A. Kay[1]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]Stanford University School of Medicine, Stanford, CA

Recombinant adeno-associated virus (AAV) vectors for genetic and acquired central nervous system (CNS) disorders continue to gain popularity exemplified by the FDA approval of Zolgensma[R], an AAV9 vector expressing the SMN1 gene from motor neurons for the treatment spinal muscular atrophy. Unfortunately, extremely high doses of the drug are required, leading to varying degrees of adverse effects, including death. A major limitation in the field is the relatively low penetration of vectors across the human blood-brain barrier (BBB). Once across the BBB, selective transduction of various cell types would also be important depending on the type of disorder being treated. In order to enhance human BBB penetration and select for CNS cell selective AAV capsids, we developed two key techniques. First, we used an *in vitro* transwell BBB system with human endothelial cells and separate layers of human iPS cell neurons and astrocytes derived from 3D organoid cultures. We then

tested a pool of 18 known AAV vectors by passaging them through our model system. The pool included AAVs known to cross the BBB more efficiently, such as AAV9 and AAV-rh.10, and those recognized not to cross the BBB (e.g AAV-DJ). The proportion of AAV-9 and AAV-rh10 capsids was enriched in the flowthrough by ~2-fold, while AAV-DJ was reduced by ~5-fold. We created a complex AAV capsid library with ~1 million variants, each containing a unique DNA barcode, using multispecies interbreeding. Three different genetic evolutionary selection schemes were carried out in astrocytes, neurons and the flow thru media. After multiple passages through the humanized BBB transwell system, we used both high-throughput single molecule capsid DNA (2.2kb) sequencing (PacBio) as well as barcode sequencing (Illumina), to identify the capsids that were enriched in the three screens. Multiple enriched AAV variants were made up of complex capsid chimeric sequences that have enhanced ability to pass through the BBB and transduction of astrocytes and/or neurons. We have validated six viral vectors from the screen for astrocytes and all six are able to cross the endothelial cell layer and transduce astrocytes 37 to 91-times better than AAV9. We also found four viral vectors from the screen for neurons that were 3.6 to 20-times better than AAV9 at crossing both the endothelial and astrocyte cell layers. These four vectors are also 1.6 to 4.0-times better than AAV9 at directly transducing iPS-derived neurons. We evaluated the capsids in mice and found several that are liver de-targeted. Our next plan is to pool the best capsids and determine their BBB penetration in non-human primates. Towards this plan, we have selected 6 of the candidates, pooled them together and established their relative BBB penetration in the transwell system, which was predicted as when each was tested individually. Our work supports the use of a human transwell system for selecting improved capsids for CNS based gene therapeutics.

54. Expanding the AAV Toolbox for Cerebellar Transduction: Identifying and Characterizing Novel Variants in Non-Human Primates and Mice

Megan S. Keiser, Yong Hong Chen, Paul T. Ranum, Xueyuan Liu, Congsheng Cheng, David Leib, Geary Smith, Amy Muehlmatt, Luis Tecedor, Beverly L. Davidson

Children's Hospital of Philadelphia, Philadelphia, PA

Introduction: New tools for gene therapy have opened the door to addressing an almost infinite number of genetic lesions, however delivering these tools to the appropriate anatomical location and cell type remains a significant bottleneck. Spinocerebellar ataxias (SCAs) are a family of hereditary movement coordination disorders broadly characterized by neurodegeneration of the cerebellum and brainstem. Here, we characterize the hindbrain tropism of newly identified capsids from an ongoing directed AAV evolution screen in nonhuman primates (NHPs) for future delivery of SCA gene therapies **Methods**: Directed evolution libraries were comprised of AAV1, AAV2, and AAV9 variants generated by addition of random 7-mer peptides into wild-type capsids. Top hits were identified following three consecutive rounds of enrichment based off pooled results from 14 individual brain regions including cerebellar cortex, deep cerebellar nuclei (DCN) and brainstem. Two capsids with enrichment in the cerebellum and brainstem were generated to express fluorescent reporters for visualization of transduction in vivo. Individual variants were delivered to NHPs or adult mice by ICV injection or direct injection in the DCN. **Results**: Following a single ICV injection, a top AAV9 variant was able to transduce Purkinje cells across multiple lobules of the cerebellum as well as a wide variety of other cerebellar cell types including Bergman glia, granule cells, and dispersed interneurons in NHP brain. Injection of the AAV9 variant ICV to adult mice revealed transduction of Purkinje cells. In addition, a top AAV2 capsid variant showed uniformly high levels of Purkinje cell expression in superficial lobules of the cerebellum. Other areas highly transduced in the adult murine brain included the motor cortex, basal ganglia, thalamic nuclei, and brainstem neuronal populations known to be affected in SCA types 1, 2, and 3. Direct parenchymal injections of our AAV2 variant to the DCN of adult mice revealed nearly complete transduction of Purkinje cells in rostral lobules of the cerebellum with positive transduction of multiple brainstem nuclei. Further testing to characterize the biodistribution profiles following different routes of administration

in adult mice and nonhuman primates is ongoing. **Conclusion**: These newly identified variants expand the utilities of AAVs to treat cell types refractory to standard AAV serotypes, and improve targeted delivery following directed or broad administration to treat multiple forms of the spinocerebellar ataxias.

55. Capsid Display of Cell-Penetrating Peptides Yields AAVs with Enhanced Brain Penetration in Both Rodents and Primates

Yizheng Yao[1], Jun Wang[1,2], Yi Liu[1], Yuan Qu[1], Kaikai Wang[1], Yang Zhang[1], Yuxin Chang[1], Zhi Yang[1], Jie Wang[1], Choi-Fong Cho[1], Fengfeng Bei[1]

[1]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA,[2]Renmin Hospital of Wuhan University, Wuhan, China

Adeno-associated viruses (AAVs) have emerged as promising vectors for gene therapy in the central nervous system (CNS). Engineering of AAV capsid has met with varying degrees of success in generating neurotropic AAV variants, as studies of several AAVs with strong CNS tropism in small model animals fail to translate to large animals such as non-human primates (NHPs). Here, we applied a rational design approach of engineering CNS-penetrating AAVs by displaying cell-penetrating peptides (CPPs) on the AAV capsid. Individual CPPs were inserted into the capsid of AAV9, and the resulting variants were screened for brain transduction after intravenous administration. Peptide sequence optimization by multiple rounds of iteration was performed for leading CPP candidates. Such design approach yielded two variants, namely AAV.CPP.16 and AAV.CPP.21, with 6-249 folds of higher efficiency than AAV9 in brain transduction across four mouse strains. Importantly, the advantage of AAV.CPP.16 over AAV9 for CNS gene delivery translates from mice to NHPs, as AAV.CPP.16

is approximately 5-fold more efficient in systemically transducing the brain in NHPs. In the meanwhile, only a modest increase of efficiency over AAV9 was observed for AAV.CPP.21 in NHPs, in contrast to its more notable brain penetration in mice. Further characterization of AAV.CPP.16 revealed the new variant transduces both neuron and astrocytes in the CNS with notable tropism to the motor neurons in particular. No capsid-associated immuno-toxicity was detected in NPHs based on behavioral monitoring and blood assays, and no cellular abnormality was observed in the dorsal root ganglion cells, which could be associated with potential AAV toxicity. Furthermore, no evidence of break-down of the blood-brain barrier (BBB) by AAV. CPP.16 was observed, and neither AAV.CPP.16 or AAV.CPP.21 binds to Ly6a, a previously identified receptor that is capable of mediating BBB crossing for certain AAV variants. Ongoing studies are further looking into the mechanisms of action on AAV.CPP.16's tropism to CNS tissues. Together, our study reports a novel CNS-tropic AAV vector AAV.CPP.16 with potential applications for gene therapy in both rodents and primates.

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56. Engineering AAV6-Based Vectors for Improved Ocular Transduction Following Intravitreal and Intracameral Injection

Sean M. Crosson[1], Shreyasi Choudhury[2], James Peterson[1], Antonette Bennett[3], Diego Fajardo[1], Andras Komaromy[4], Mavis Agbandje-McKenna[3], Sanford L. Boye[5], Shannon E. Boye[1]

[1]Pediatrics, Cell and Molecular Therapy, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Florida, Gainesville, FL,[3]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,[4]Small Animal Clinical Sciences, University of Michigan State, East Lansing, MI,[5]Pediatrics, Powel Gene Therapy Center, University of Florida, Gainesville, FL

Purpose: The transduction and tropism of AAV in the eye is dependent on vector administration route. Intravitreal injection (IVI) is less surgically complex than a subretinal injection but requires AAVs that traverse the inner limiting membrane, a behavior that relies on the capsid's ability to bind heparan sulfate proteoglycan (HSPG). Transduction of trabecular meshwork (TM) in the anterior segment similarly depends on HSPG binding. The purpose of our study was to rationally engineer AAV6-based vectors for improved transduction of retina and TM following intravitreal or intracameral (ICI) injection, respectively. **Methods:** We evaluated transduction of HEK293, 661W, and rMC-1 cells by AAV1 and AAV6 using flow cytometry. We generated a series of AAV6-based mutants containing previously described proteasomal avoidance (PA) mutations to surface-exposed S, T, and/or Y residues [AAV6(S551V+S663V), AAV6(T492V+S663V), and AAV6(T492V+Y705F+Y731F)] and evaluated their performance in rMC-1 cells. WT mice were IVI with 1E9 vg of AAV6(T492V+Y705F+Y731F), 'aka' AAV6(3pMut), AAV6, or benchmark shH10(Y445F), and evaluated at 6 weeks post-injection (p.i.) by fundoscopy and immunohistochemistry (IHC). Additional variants with increased HSPG affinity or PA, AAV6(3pMut)D532N, AAV6(3pMut)D532R, and AAV6(4pMut) were IVI in mice and evaluated as above. Heparin binding assays were performed using heparin-conjugated agarose beads and gravity flow columns. WT rats received ICI injections of AAV6, AAV6(3pMut), or AAV6(3pMut) D532N (2E9 vg) and the TM, lens, and cornea were imaged by IHC 4 weeks p.i. **Results:** AAV6 variants harboring PA mutations transduced rMC-1 cells with significantly higher efficiency than unmodified AAV6, with AAV6(3pMut) having the highest transduction. In IVI mice, AAV6(3pMut) outperformed both AAV6 and shH10(Y445F) while all had similar tropism for Müller glia (MG) and retinal ganglion cells (RGCs). Addition of D532N and D532R mutations to AAV6(3pMut) increased heparin affinity, more so for AAV6(3pMut) D532R; while the addition of a 4th PA mutation [AAV6(4pMut)] slightly decreased heparin affinity. In IVI mice, AAV6(3pMut)D532N transduction was comparable to AAV6, while AAV6(4pMut) had reduced transduction and AAV6(3pMut)D532R did not transduce. All infectious variants had MG and RGC tropism. AAV6(3pMut)

D532N transduced TM most efficiently, followed by AAV6, then AAV6(3pMut). **Conclusions:** Incorporation of PA mutations onto AAV6 enhances retina transduction via IVI. However, addition of a 4th PA mutation [AAV6(4pMut)] diminished retinal transduction, indicating PA alone is not the sole determinant of transduction via IVI. This reduction is likely attributed to this capsid's decreased HSPG affinity, which may be influenced by the hydrophobic nature of PA mutations. Conversely, retinal transduction by AAV6(3pMut) D532N was comparable to AAV6 but worse than AAV6(3pMut) via IVI, suggesting that increased HSPG affinity counteracts the effects of PA mutations. In TM, AAV6(3pMut)D532N transduction was higher than AAV6 and AAV6(3pMut) suggesting that increased HSPG affinity is a main a driver of TM transduction, rather than PA. Overall, these results suggest that transduction of ocular tissues by AAV requires a balance between the capsid's HSPG affinity and PA.

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57. *ST3GAL5* Gene Replacement in CNS Restores Gangliosides Production and Improves Survival in a Mouse Model of GM3 Synthase Deficiency

Huiya Yang[1], Karlla Brigatt[2], Jia Li[1], Kazuhiro Aoki[3], Michael Tiemeyer[3], Robert H. Brown[4], Dan Wang[a5], Kevin A. Strauss[a2], Guangping Gao[a1]

[1]Horae Gene Therapy Center and Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[2]Clinic for Special Children, Strasburg, PA,[3]Complex Carbohydrate Research Center and The Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA,[4]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[5]Horae Gene Therapy Center and RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

GM3 synthase (ST3GAL5) deficiency is caused by biallelic mutations of the *ST3GAL5* gene, and results in complete systemic deficiency of GM3 and its downstream a- and b-series cerebral gangliosides (GM1, GD1a, GD1b, and GT1b). The clinical

phenotype is characterized by hearing and visual impairment, intractable epileptic encephalopathy, irritability, failure to thrive, progressive microcephaly, developmental stagnation, and reduced survival. GM3 synthase deficiency follows an autosomal recessive pattern with an estimated incidence of approximately 1 per 1,200 births in Old Order Amish communities of North America. No disease-modifying treatment is currently available. Here, we hypothesize that *ST3GAL5* replacement therapy targeted to the central nervous system (CNS) could drive ST3GAL5 expression in neurons and oligodendrocytes, restore the endogenous production and trafficking of cerebral gangliosides, and thereby rescue the severe neurodevelopmental phenotype. We first used lentivirus-mediated gene replacement to transfer human h*ST3GAL5* cDNA *in vitro*, which restored GM3 production in patient-derived fibroblasts. More importantly, h*ST3GAL5* replacement in patient iPSC-derived cortical neurons reconstituted GM3 as well as its major downstream a- and b-series brain gangliosides. These *in vitro* data indicate the strong therapeutic potential of human *ST3GAL5* cDNA gene replacement. Next, to prove our hypothesis *in vivo*, we packaged h*ST3GAL5* constructs into AAV9 capsids. To examine the safety and efficacy of transgene expression, we first delivered AAV vectors via intracerebroventricular (ICV) administration to wild type (WT) neonatal C57BL/6 mice. We observed robust transgene expression in CNS without short-term vector-associated toxicity. Furthermore, to assess the therapeutic efficacy of *ST3GAL5* gene replacement, we delivered AAV9-h*ST3GAL5* vectors in *St3gal5/B4galnt1* double knock-out (DKO) mice, which are unable to synthesize any cerebral gangliosides and mirror the phenotype of human GM3 synthase deficiency. Direct ICV treatment of neonatal DKO mice restored the production of GD1a, GD1b, and GT1b, improved physical growth, and extended median survival from 20 days to more than 5 months. To our surprise, administration of AAV9-h*ST3GAL5* via systemic injection triggered acute liver damage 2-days post injection that culminated in animal death. At the mRNA level, h*ST3GAL5* expression was 30-fold higher than normal in liver, which may be the cause of the liver damage. We therefore modified our vector design from a ubiquitous to neuronspecific promoter, which alleviated acute liver toxicity. In ongoing studies, we are further improving rAAV-based h*ST3GAL5* replacement vectors and testing their safety and efficacy in *St3gal5*-/- and *St3gal5B4galnt1* double knockout mice. [a]Co-corresponding authors

58. CRISPR/Cas9 Strategies to Treat Spinocerebellar Ataxia Type 1

Kelly Fagan[1], Megan Keiser[2], Beverly Davidson[2]

[1]University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that causes progressive loss of motor coordination, respiratory issues and eventual death. SCA1 is caused by expansion of the polyglutamine repeat region in the *ATXN1* gene. Normal *ATXN1* alleles contain 6-42 CAG trinucleotide repeats with interspersed CAT nucleotides, while disease alleles have an uninterrupted CAG region with 39-100+ repeats. The mechanism of SCA1 pathogenesis is unknown; however, some features of the disease include neuronal degeneration and formation of toxic mutant ATXN1 (mATXN1) nuclear inclusions. Although mATXN1 is expressed ubiquitously, it affects primarily Purkinje cells (PCs). There are currently no treatment options for SCA1. We hypothesize that CRISPR-Cas9 editing of *ATXN1* will reduce mutant ATXN1 and be therapeutically beneficial. We designed two different strategies to reduce ATXN1; the first uses a single guide RNA (gRNA) to target near the exon-exon junction to induce nonsense mediated decay, while the second approach employs a dual guide system to delete the CAG repeat region. gRNAs were optimized in vitro, with each approach significantly reducing *ATXN1* expression. The single guide approach reduced *ATXN1* mRNA levels by 40-45% ($p \leq 0.02$) and protein by approximately 20% ($p \leq 0.01$) and the dual guide approach reduced levels of mRNA and protein levels by 70-75% ($p < 0.001$) and 45-65% ($p \leq 0.03$), respectively. For testing in vivo, SCA1 mice were crossed to *sp*Cas9 transgenic mice; SCA1 mice express human *ATXN1* with an expanded 82 CAG repeats in Purkinje cells, and show progressive motor deficits and neuropathology. Recombinant AAVs (rAAVs) expressing the optimized gRNAs (EE2 and EE4) from the exon-exon strategy were delivered directly to the deep cerebellar nuclei of 5-week-old SCA1/*sp*Cas9 mice for transduction of Purkinje cells. The EE2 gRNA reduced protein and mRNA levels by 55% ($p=0.05$) and 50% ($p=0.02$), respectively and EE4 decreased ATNX1 protein and mRNA levels by 10% ($p=0.9$) and 20% ($p=0.07$) compared to saline injected controls. The EE2 gRNA more effectively reduced ATXN1 levels, and studies are in progress to assess its impact on SCA1 mice phenotypes.

59. Rescue of Molecular and Motor Phenotypes in CGG Knock-In Mice with CRISPR Mediated Deletion of the Trinucleotide Repeat

Carolyn M. Yrigollen[1], Laura Ohl[1], Euyn Lim[1], ShuJuan Zheng[2], Kasey Brida[3], Yong Hong Chen[1], Alejandro Mas Monteys[1], Beverly L. Davidson[1]

[1]CCMT, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Temple University, Philadelphia, PA,[3]University of Alabama at Birmingham, Birmingham, AL

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder that is caused by a premutation allele (55-200 CGG repeats) in the 5' untranslated region of the *FMR1* gene. Gene-editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has previously been shown to delete the CGG repeats of *FMR1 in vitro*, but this has not yet been reported *in vivo*, nor the molecular and phenotypic effects of such a treatment strategy. We evaluated a Cas9 based therapy for its ability to correct *FMR1* trinucleotide repeats in the *Fmr1* CGG knock-in (KI) mouse with approximately 130 CGG repeats. These mice exhibit motor and memory impairments and are used to model FXTAS. Two guideRNAs (gRNAs) were used to target the CGG repeats for deletion upon Cas9 mediated cleavage. Dual AAV vectors containing Cas9 and the gRNAs were injected into mouse striatum and tissues harvested three weeks post injection. Isolated DNA

showed complete or partial deletion of the CGG repeats along with deletion of adjacent nucleotides upstream and downstream of the target site. The transcriptional start site and the start codon of *Fmr1*, elements required for functional gene expression, remained intact. Striatal expression of *Fmr1* in untreated KI mice had a 3-fold upregulation compared to WT littermates, consistent with the known upregulation of this transcript with CGG repeat expansion. However, KI mice treated with targeting gRNAs/ Cas9 had significantly lower *Fmr1* expression than untreated mice ($p < 0.0001$), with levels similar to WT. Treated and untreated mice from both genotypic groups had Fmrp levels that did not differ significantly from each other, suggesting the gene editing that corrected the regulation of *Fmr1* mRNA levels did not hinder synthesis of the encoded protein in *Fmr1* CGG knock-in mice. To investigate whether rescue of *Fmr1* transcriptional regulation would result in phenotypic rescue, neonatal mouse pups were injected at P0 to P1 bilaterally into the cerebral ventricles with the dual AAV vectors. Motor performance was evaluated at 12-15 weeks, 28-30 weeks and 52-54 weeks of age by accelerating rotarod. Motor impairment occurs in KI mice by 28 weeks, and results in quicker falls from the apparatus than is observed in WT littermates. Mice that were treated with the CRISPR constructs performed better than untreated knock-in mice, $p = 0.0041$ and were not statistically different their WT littermates at 28-30 weeks of age. At the late stage rotarod the *Fmr1* treated mice continued to perform similarly to the WT animals, but statistical differences were no longer observed between treatment groups. Additional analysis of behavioral and molecular data is ongoing.Our study is the first to demonstrate *in vivo* editing of expanded CGG repeats in *Fmr1* using CRISPR. Here we have shown that our guides enable safe excision, removing the expanded repeat, rescuing expression of *Fmr1* mRNA, and maintaining Fmrp synthesis. Importantly, we show *in vivo* CRISPR mediated editing of the *Fmr1* trinucleotide repeat has a therapeutic benefit, rescuing motor deficits present in aged KI mice. These results indicate that

CRISPR-mediated gene editing has the potential to ameliorate the degenerative pathology present in FXTAS and further development of this strategy for treatment of FXTAS and other Fragile X-associated disorders is necessary.

60. Transthyretin Gene Therapy as a Modulator of Alzheimer's Disease Progression

Ana Rita Batista[1], Robert M. King[2], Thais Hernandez[1], Monique Otero[1], Paola Rodriguez[1], Matthew J. Gounis[2],
Miguel Sena-Esteves[1]

[1]Dep of Neurology and Horae Gene Therapy Center, University of Massachussets Medical School, Worcester, MA,[2]NECStR, University of Massachussets Medical School, Worcester, MA

Epidemiological studies and work in transgenic animals suggest that transthyretin (TTR) levels in plasma and/or CSF may modulate Alzheimer's disease (AD) presentation and/or progression. AD patients exhibit lower levels of TTR in CSF compared to agematched healthy individuals. Similarly, genetic reduction of TTR in mouse models of AD is associated with earlier onset and more severe phenotypes. The current study was designed to determine the potential of somatic AAV mediated TTR expression to modulate disease presentation in AD.We designed an AAV9 vector to express TTR specifically in liver and choroid plexus using a TTR promoter to drive transgene expression. Two-month-old male and female 5XFAD mice were injected systemically with 2E12 gc AAV9-TTRp-TTR^{T119M} (n=12M+12F), or PBS as controls (n=12M+12F). At nine months of age animals underwent neurobehavioral tests to assess cognitive function, and 3D isotropic T2-weighted MRI to evaluate differences in brain volume. Post-mortem assessments include histological evaluation of plaque burden, quantification of Abeta levels in different brain structures, quantification of neurofilament light-chain (NfL) in serum, among others still on-going.Cognitive function was assessed through measurement of working and spatial memory using forced and spontaneous alternation tasks in the Y-maze, and novel object recognition tests. We observed behavioral improvements in the females only, namely regarding in the percent time interacting with novel object. General locomotion and anxiety were evaluated using the open field test, where AAV treated females presented higher locomotor activity and increased exploratory behavior. Interestingly, no differences were observed in males. Surprisingly, MRI analysis revealed increased CSF volume in all treated animals compared to controls, with females showing larger differences.Histological analysis of plaque burden using 6E10 antibody staining showed a remarkable difference in the treated females in comparison to controls. Analysis of the males is still ongoing.Our initial results show that systemic delivery of an AAV9 vector that expresses TTR in appropriate tissues improves cognitive function, locomotor activity and anxiety symptoms in 5XFAD mice, but this benefit is more noticeable in females. The unexpected increase in CSF volume may be related to increased TTR production by the choroid plexus of AAV treated animals, and additional experiments are ongoing to determine the cause of this outcome.Overall, this new AAV vector represents a therapeutic platform to study biologically relevant questions about the role of TTR in AD.

61. CRISPR/Cas9-Mediated Excision of ALS/FTD-Causing Hexanucleotide Repeat Expansion in C9ORF72 Rescues Major Disease Mechanisms *In Vivo* and *In Vitro*

Katharina E. Meijboom[1], Abbas Abdallah[1], Nicholas Fordham[1], Hiroko Nagase[1], Tania F. Gendron[2], Gopinath Krishnan[3], Tomás Rodriguez[3], Alireza Edraki[3], Meghan Blackwood[1], Erik Sontheimer[3], FenBiao Gao[3], Robert H. Brown[3], Zane Zeier[4], Christian Mueller[1]

[1]Gene Therapy Center, UMASS Medical School, Worcester, MA,[2]Mayo Clinic, Jacksonville, FL,[3]UMASS Medical School, Worcester, MA,[4]University of Miami, Miami, FL

A hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{24+}$ in the *C9ORF72* gene is the most common genetic cause of

amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Both are fatal neurodegenerative diseases with no current approved treatments that significantly slow disease progression or extend life expectancy. Several hypotheses have emerged to explain how this HRE causes neuronal death, including haploinsufficiency, sequestration of RNAbinding proteins in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide production. In the present study we used a CRISPR/ Cas9 gene-editing approach to remove the HRE from the C9ORF72 genomic locus, designing guide RNAs (gRNAs) flanking the HRE, and delivered Cas9 and gRNAs via adeno-associated virus serotype 9 (AAV9) vectors. Here, we demonstrate successful excision of the HRE in C9ORF72 in primary cortical neurons and in the brains of three mouse models containing the C9ORF72 expanded HRE (ranging from 500-600 repeats) as well as in iPSC motor neurons and brain organoids (450 repeats). This resulted in a reduction of RNA foci and poly-dipeptides and a rescue of haploinsufficiency, the major hallmarks of C9-ALS/FTD. This work is, to our knowledge, the first to demonstrate an *in vivo* therapy that addresses both toxic gains-offunction conferred by to mutant RNAs and polydipeptides, but also haploinsufficiency, making this an extremely attractive therapeutic approach to these diseases.

62. C9ORF72 Variant-Specific RNA Interference Rescues C9-ALS/FTD Molecular Hallmarks *In Vivo* and *In Vitro*

Katharina E. Meijboom, Nicholas Fordham, Meghan Blackwood, Gabriella T. Cabrera, Robert H. Brown, Christian Mueller

UMASS Medical School, Worcester, MA

Amyotrophic lateral sclerosis (ALS) is a terminal neurodegenerative disease that affects upper and lower motor neurons, causing progressive muscle weakening and respiratory failure. A hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{23+}$ in the intronic region of C9ORF72, contributes to 40% of familial and 10% of total ALS cases. Up to 50% of patients with this expansion also develop frontotemporal dementia (FTD). The major proposed disease mechanisms behind this HRE include haploinsufficiency, RNA binding proteins sequestration in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide production. Both C9ORF72 ALS and FTD are aggressive diseases with no treatments to significantly slow disease progression or extend life expectancy. We are developing two AAV-RNAi gene therapy approaches using artificial microRNAs packaged in AAVrh10 to: (1) specifically target C9ORF72 mRNA variants that contain the HRE (variant (V) 1 and V3) and preserve the most abundant variant, V2, which does not contain the HRE, to avoid haploinsufficiency; (2) specifically target the intron containing the HRE, thereby only targeting incorrectly spliced transcripts. Using approach (1) we significantly lowered levels of V1 in treated primary neurons of C9ORF72 BAC-transgenic mice expressing an expanded repeat (500) compared to untreated groups, while overall C9ORF72 levels remained unaffected. We next treated adult C9ORF72 BAC-transgenic mice through striatal injections and similarly reduced levels of V1 mRNA containing the HRE, but not in V2 in the striatum. This reduction of HRE-containing transcripts by artificial miRNAs resulted in a decrease of GP poly dipeptides and RNA foci. Our preliminary studies using approach (2) have demonstrated a large, significant decrease of intron-containing transcripts. Future experiments will test the efficacy of both therapeutic approaches *in vivo* and in patient-derived motor neurons to eliminate existing - and prevent the formation of - toxic dipeptides and RNA foci formation, to ultimately rescue motor neuron pathology in ALS and FTD.

63. Restoration of *Scn1a* Expression after Symptom Onset in a Novel Model of Dravet Syndrome Rescues Seizures and Behavioral Alterations

Gaia Colasante[1], Nicholas Valassina[1], Simone Brusco[1], Linda Serra[1], Alessia Salamone[1], Gabriele Lignani[2], Vania Broccoli[1]

[1]Division of Neuroscience, Ospedale San Raffaele, Milan, Italy,[2]Division of Neuroscience, UCL, London, United Kingdom

Dravet syndrome is a severe epileptic encephalopathy that begins during the first year of life and leads to severe cognitive and social interaction deficits. It is mostly caused by heterozygous loss-of-function mutations in the SCN1A gene, which encodes for the alpha-subunit of the voltage-gated sodium channel (Nav1.1) and is responsible mainly of GABAergic interneuron excitability. While different therapies based on the upregulation of the healthy allele of the gene are being developed, the dynamics of reversibility of the pathology are still unclear. In fact, whether and to which extent the pathology is reversible after symptom onset and if it is sufficient to ensure physiological levels of Scn1a during a specific critical period of time are open questions in the field and their answers are required for proper development of effective therapies.We generated a novel *Scn1a* conditional knock-in mouse model (Scn1a floxSTOP) in which the endogenous Scn1a gene is silenced by the insertion of a floxed STOP cassette in an intron of Scn1a gene; upon Cre recombinase expression, the STOP cassette is removed, and the mutant allele can be reconstituted as a functional Scn1a allele. In this model we can reactivate the expression of Scn1a exactly in the neuronal subtypes in which it is expressed and at its physiological level. Those aspects are crucial to obtain a final answer on the reversibility of DS after symptom onset. In fact, for all the gene therapy approaches that are being explored in different laboratories, several factors converge on the final therapeutic efficacy: the real

ability of the strategy in boosting Scn1a gene expression at single cell level, the targeting of the correct neuronal subtype and the total number of cells reached by the treatment.We exploited this model to demonstrate that global brain re-expression of the Scn1a gene when symptoms are already developed (P30) led to a complete rescue of both spontaneous and thermic inducible seizures and amelioration of behavioral abnormalities characteristic of this model. We also highlighted dramatic gene expression alterations associated with astrogliosis and inflammation that, accordingly, were rescued by Scn1a gene expression normalization at P30. Moreover, employing a conditional knock-out mouse model of DS we reported that ensuring physiological levels of Scn1a during the critical period of

symptom appearance (until P30) is not sufficient to prevent the DS, conversely, mice start to die of SUDEP and develop spontaneous seizures. These results offer promising insights in the reversibility of DS and can help to accelerate therapeutic translation, providing important information on the timing for gene therapy delivery to Dravet patients.

AAV Biology, Engineering, Immunology and Animal Modeling

64. A Multi-Mechanistic Anti-Angiogenic AAV Gene Therapy Product Candidate, 4D-150, for the Treatment of Wet Age-Related Macular Degeneration (wAMD) and Diabetic Macular Edema (DME): Intravitreal Biodistribution, Transgene Expression, Safety and Efficacy in Non-Human Primates

Peter J. Francis[1], Peter J. Francis[1], Christian Burns[2], Paul Szymanski[2], Meredith Leong[3], Nima Mohaghegh[4], Christopher Schmitt[5], Austin Klein[5], Roxanne H. Croze[5], Melissa A. Calton[3], Devi Khoday[6], Melissa Kotterman[2], Katherine Barglow[7], David Schaffer[1], David Kirn[1]

[1]4D Molecular Therapeutics, Emeryville, CA,[2]Discovery & Engineering, 4D Molecular Therapeutics, Emeryville, CA,[3]Translational Medicine, 4D Molecular Therapeutics, Emeryville, CA,[4]Bioinformatics, 4D Molecular Therapeutics, Emeryville, CA,[5]HCDM, 4D Molecular Therapeutics, Emeryville, CA,[6]Upstream Process Development, 4D Molecular Therapeutics, Emeryville, CA,[7]Process and Analytical Development, Characterization, 4D Molecular Therapeutics, Emeryville, CA

Introduction Major blinding conditions such as wAMD and DME are characterized by abnormal retinal angiogenesis resulting in leakage, hemorrhage and scarring with consequent vision loss. We have designed an intravitreally-delivered multi-mechanistic anti-angiogenic gene therapy (4D-150) which could prove an attractive alternative to currently-approved biologics by eliminating the need for frequent injections combined with the potential for improved efficacy. 4D-150 is comprised of the retina targeted and evolved intravitreal vector R100 and is engineered to deliver the aflibercept protein sequence (anti-VEGF A&B, Placental growth factor, PlGF) plus a second

sequence encoding ddRNAi targeting the angiogenic factor VEGF C. In our preliminary *in vitro* experiments, we have shown dose-related expression in human iPSC-derived retinal cells and demonstrated functional activity. **Methods** We assessed the in vivo efficacy of R100. anti-VEGF gene therapy (a 4D-150 prototype) in the validated NHP laser-induced choroidal neovascularization (CNV) model of wAMD across a broad range of therapeutically relevant doses. NHP were dosed IVT with the 4D-150 prototype and six weeks post-administration, underwent retinal laser photocoagulation to induce CNV lesions. After two and four weeks, the numbers of clinically relevant grade IV CNV lesions were assessed by fluorescein angiography. Anti-VEGF protein was assessed by ELISA over 12 months by serial in vivo aqueous fluid samples. We subsequently performed a study in nonhuman primates (NHP) to assess the safety of 4D-150 and to measure expression of the aflibercept protein (retina and ocular fluids) and expression of VEGFC RNAi in the retina. **Results** In the NHP CNV model, the 4D-150 prototype resulted in complete suppression of grade IV CNV lesions (0/72) in treated eyes compared to vehicle (19/72) at doses as low as 1E11vg/eye ($p<0.0001$). Measurable and dose-dependent anti-VEGF protein was detected in aqueous fluid as early as 14 days post dosing and sustained through 12 months. There were no ocular or systemic toxicities at any dose tested. Specifically, there was no evidence of chronic intraocular inflammation (uveitis). Single IVT administration of 4D-150 resulted in high levels of ocular and retinal aflibercept protein together with highly robust VEGF C RNAi expression, without evidence of toxicity. **Conclusion** 4D-150 is a multi-mechanistic antiangiogenic gene therapy that can be delivered by the simple and safe intravitreal route of administration. 4D-150 was designed with the goal of improved efficacy over single mechanism anti-angiogenic approaches by inhibiting multiple VEGF isoforms, as well as PlGF, within the retina. An intravitreal anti-VEGF prototype of 4D-150 resulted in efficacy and safety through 12 months in the NHP CNV model. Intravitreal 4D-150 resulted in sustained and high levels of functional aflibercept and anti-VEGF C RNAi. 4D-150 holds potential for the intravitreal treatment of wAMD and DME.

65. Evolving Synthetic AAV Variants for Genome Editing in Immune Cell Populations

Jonathan Ark[1], William Nyberg[2], Patrick Havlik[3],

[1] Molecular Genetics and Microbiology, Duke University, Durham, NC,[2]Parker Institute for Cancer Immunotherapy, UCSF, San Francisco, CA,[3]Surgery, Duke University, Durham, NC

The immune system is an important target for genetic manipulation, with implications for cancer immunotherapy using T or NK cell populations, correction of monogenic blood disorders using hematopoietic stem cell populations or plasma cell-based therapies. A majority of approaches to achieve homology directed repair ex vivo are performed using AAV serotype 6 to deliver the single stranded DNA repair or donor template. However, significant room for improvement with regard to cellular targeting and efficiency of donor template or transgene delivery exists. To achieve such, we adopted our structuredriven evolution approach to engineer AAV capsid libraries that can transduce primary T-cells, B-cells, NK cells, monocytes/macrophages and hematopoietic stem cells of human and mouse origin. Upon cycling, newly evolved AAV variants were significantly enriched by

Angela To[2], Justin Eyquem[2], Aravind Asokan[3]
> 1000 fold from the parental library. Highly conserved structural epitopes were mapped onto the capsid surface of lead capsid candidates, but amino acid residues were distinct for different cell lineages and with human vs murine origin as well. To assess the potential for improved transduction, we are evaluating lead AAV capsid variants in different human and mouse immune cell lineages. Notably, a human T cell evolved variant Ark312 and a mouse T cell variant Ark313 displayed 10-20 fold higher transduction efficiency in human T cells and in mouse T cells, 100-1000 fold improved transduction at low multiplicities of infection (MOI) showing a 100-1000 fold increased mean fluorescence index (MFI) with 40-fold increase in % transduced cells. Neither AAV variant infected immune cells from the other species, demonstrating host selectivity. When used to deliver an HDR/ donor template, Ark312 significantly increased knock-in frequency compared to AAV6 across a range of incubated viral titers. Remarkably, Ark313 enabled highly efficient HDR in primary mouse T cells with a knockin frequency reaching <25% at an MOI of 3e3 and above 50% at 1e5, whilst wtAAV6 afforded less than 10% at the highest MOI. These synthetic AAV variants and others under evaluation have the potential to enable greater ex vivo knockin efficiencies in a wide spectrum of immune cell-based therapies and ultimately help pave the way for immunoengineering in vivo.

66. Real Time Blood Brain Barrier Disruption in a Multi-Species Model

AR Batista[1], RM King[2], WC Baker[1], MJ Gounis[2], H. Gray-Edwards[1], M. Sena-Esteves[1]
[1]UMASS, Worcester, MA,[2]NECStR, UMASS, Worcester, MA

Central nervous system (CNS) transduction by gene therapy is highly efficient upon local delivery. However, local CNS delivery is invasive and involve complex neurosurgical procedures that carry considerable risk for patients. Systemic delivery is the simplest route to achieve broad distribution of therapeutics in CNS, but they have to cross the blood brain barrier (BBB). Several approaches have been used to enhance the efficiency of AAV CNS gene transfer, including use of mannitol or focused ultrasound to temporarily disrupt the BBB, and the development of new AAV capsids. New AAV capsids developed by in vivo capsid evolution proved remarkably efficient for CNS gene delivery in mice, but the lack of superiority to AAV9 in non-human primates (NHP) raised questions about translatability and stopped further development as a new platform for human trials in neurological diseases. Methods that facilitate a transient opening of the BBB allowing for successful systemic delivery of AAV gene therapy, and the subsequent closing of the BBB, so that its function remains intact, are vital. Here we present the application of a BBB disruptive peptide, K16ApoE, that we show to achieve this transient BBB opening in multiple species.Initial studies were performed in young adult BALB/ cJ mice by co-injecting in the tail vein 5E11 vg AAV vector encoding Firefly luciferase (FLuc) with peptide, or injection of K16ApoE alone followed by AAV 30 min later. At 3-weeks post-injection, FLuc activity was measured in CNS and liver. In a second phase, we evaluated the BBB-disrupting properties of K16ApoE in other species by MRI using dynamic gadolinium (Gd) enhancement. Three different species were used: mouse, sheep and NHP, in a paired fashion, where one animal in each pair received a co-injection of 0.1 mmol/Kg Gd and K16ApoE and the other Gd only. The MR sequence was the same for all animals: baseline 3D T1W MPRAGE for structural imaging, dynamic 2D T1W imaging with a dynamic time of 20 sec for a total of 1 hour, and finally post injection repeat of the 3D T1W MPRAGE. Co-injection of AAV9-FLuc with K16ApoE enhanced FLuc activity in brain by ~100 fold compared to AAV9-FLuc alone, ~1,000 fold with AAVrh10 and ~10 fold with AAV2, a capsid that otherwise would not reach the brain when injected systemically. When we separated the administration of AAV9 and peptide, there were no differences in FLuc activity compared to control animals. For the second phase of the experiment, animals imaged during co-injection of Gd and K16ApoE showed higher levels of maximum signal enhancement within the brain compared to those that only received Gd (Figure 1). The contrast enhancement was observed at 2-3 min post injection and reached maximum after approximately 20 min, whereby the signal remained constant for the remainder of the imaging. In the animals that only received Gd, the signal was seen to increase immediately, consistent with contrast in the vascular system, and then return to baseline levels in less than 3 min. All animals recovered from anesthesia normally with no apparent side effects.K16ApoE peptide is able to transiently open the BBB and allows for transport of different AAV capsids as well as Gd over a span of less than 30 min, when compared to animals that did not receive peptide where there was little to no crossing of the BBB. The use of this peptide was well tolerated by all the species. The combination of systemic AAV gene therapy with a peptide that temporarily disrupts the BBB is a powerful approach to potentiate therapies for neurological diseases.

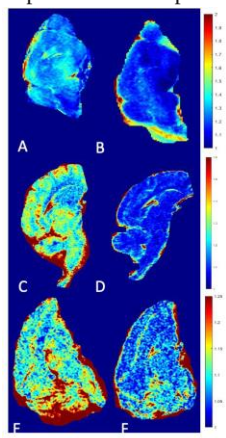


Figure 1:
Maximum enhancement maps for the dynamic T1W MRI. First column (A,C,E): Animals co-injected with gadolinium and K16ApoE. Second column (B,D,F): Animals injected with gadolinium alone. In the top row (A,B) shows mouse brains, the second row (C,D) shows sheep brains, and the final row (E,F) shows NHP brains. In all the species shown, the animals that received injection of gadolinium combined with peptide show greater levels of maximum enhancement over the brain parenchyma. In the animals that received gadolinium alone, only the choroid plexus (*) showed enhancement.

67. AAV2:2.retro-Mediated Delivery of Mutant Huntingtin throughout Cortico-Basal Ganglia Circuitry Leads to the Progressive Development of Motor and Cognitive Decline, along with Microstructural Changes in White and Gray Matter, in a Novel Rhesus Macaque Model of Huntington's Disease

Alison R. Weiss[1], William A. Liguore[1], Kristin Brandon[1], Xiaojie Wang[1,2], Zheng Liu[1,2], Jackie

Domire[1], Dana Button[1], Christopher D. Kroenke[1,2,3], Jodi L. McBride[1,3,4]

[1]Division of Neuroscience, Oregon National Primate Research Center/ OHSU, Beaverton, OR,[2]Advanced Imaging Research Center, OHSU, Portland, OR,[3]Department of Behavioral Neuroscience, OHSU, Portland, OR,[4]Neurology, OHSU, Portland, OR

To create a nonhuman primate model of the neurodegenerative brain disorder, Huntington's disease (HD), we injected adult rhesus macaques into the caudate and putamen with a 1:1 mixture of AAV2 and AAV2.retro expressing a fragment of m*HTT* bearing 85 CAG repeats. Previous work by our lab shows that this injection strategy leads to the expression of mutant HTT protein, and the formation of hallmark HTT+ inclusions, throughout the striatum as well as dozens of cortical and subcortical brain structures due to the strong retrograde capability of AAV2.retro. Here, we queried the disruption of cortico-basal ganglia circuitry for 14-months post-delivery of this mHTT construct (HTT85Q, n=6), a control HTT construct bearing 10 CAG repeats (HTT10Q, n=6) or PBS buffer (n=5). We characterized the emergence of motor and cognitive phenotypes to link behavioral changes with disruptions in cortico-basal ganglia circuitry using multimodal neuroimaging techniques including diffusion tensor imaging (DTI) and T1/T2-weighted magnetic resonance imaging (MRI). To achieve this, we evaluated animals using complex behavioral tasks to assess fine motor coordination (Lifesaver Retrieval Task), gross motor function (NHP-specific neurological rating scale), working memory (Spatial Delayed Response) and object recognition (Delayed Non-Match to Sample) to complement our neuroimaging battery. Compared to buffer and HTT10Q treated controls, animals treated with AAV2:2retro-HTT85Q showed a progressive development of mild orofacial dyskinesia, aberrant forelimb posture, forelimb chorea, incoordination, hindlimb slowness (bradykinesia) and/or tremor, which were exacerbated by the dopamine receptor agonist apomorphine. Compared to baseline measures, control animals also performed better on the Lifesaver Retrieval Task than HTT85Q animals. Moreover, compared to controls, HTT85Q animals exhibited impaired spatial working memory, but preserved object recognition. Voxel-based DTI analysis revealed many white and gray matter regions with alterations in fractional anisotropy in HTT85Q animals, suggesting that mHTT expression resulted in microstructural changes throughout the cortico-basal ganglia circuit. Similar voxel-based approaches are currently being applied to the T1w/T2w images using tensor-based morphometry to address whether HTT85Q leads to localized changes in brain volume. Additional efforts are also underway using positron emission tomography (PET) imaging to explore perturbations in the dopamine system (F18-Fallypride) as well as regional changes in glucose metabolism (F18-FDG). These data

demonstrate the feasibility of generating AAV-based models of HD in nonhuman primates that exhibit the hallmark motor and cognitive behavioral phenotypes, as well as neuropathological manifestations, of HD. Using a combination of AAV2 and AAV2.retro allowed for the expression of mHTT throughout the cortico-basal ganglia circuit, versus just the striatum, leading to the creation a NHP model of this disease that more closely depicts the neuropathology observed in human HD patients. Therefore, our studies also set the stage for developing novel biomarkers of disease manifestation, as well as using this gene delivery approach to test promising therapeutics in our model.

68. Investigating Mechanisms of Variability of AAV5-hFVIII-SQ Expression in Mice

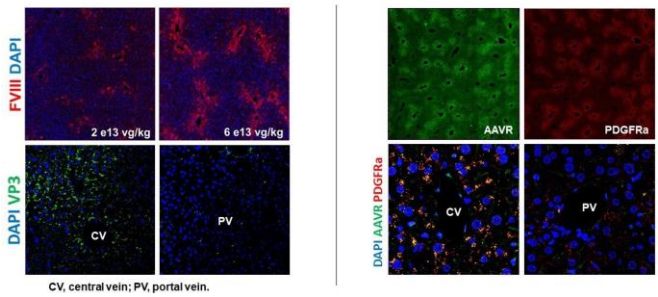
Su Liu, Bridget Yates, Lisa Razon, Britta Handyside, Lening Zhang, Ryan Murphy, Jessica Felix, Catherine Vitelli, Weiming Zhang, Cheng Su, Sherry Bullens, Stuart Bunting, Sylvia Fong

BioMarin Pharmaceutical Inc., Novato, CA

Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is an investigational gene therapy under development for the treatment of severe hemophilia A. Significant intersubject variability has been observed in AAV5hFVIII-SQ gene expression outcomes across species and trials. We systematically investigated host factors in mice that may affect transgene expression at transduction, transcription, and protein translation/secretion. Male C57BL6 mice were administered a single vector dose ($1x10^{13}$ to $2x10^{14}$ vg/kg) intravenously. Levels of liver FVIII DNA, RNA, and protein; plasma FVIII protein; and various markers were analyzed. At doses of 1-$3x10^{13}$ vg/kg (producing therapeutic levels), significant correlation was noted between levels of liver vector DNA and FVIII protein ($r = 0.7312$, $P < 0.0001$), suggesting that hepatocyte transduction (from vector uptake, trafficking, genome processing to retention) may be an important contributing factor. At higher doses of vector ($\geq 6x10^{13}$ vg/kg), a strong correlation between levels of liver Grp78, a chaperone protein responsible for folding and secreting proteins, and plasma FVIII protein levels ($r = 0.7613$; $P < 0.0001$) was observed, suggesting individuals who have a greater intrinsic ability to fold FVIII protein may secrete higher levels of mature protein into circulation. While neutralizing AAV5 antibodies can inhibit transduction, other non-antibody soluble factors may also impact transduction. For example, levels of liver vector DNA positively correlated with predosing serum cholesterol ($r = 0.491$; $P = 0.0279$) and progesterone levels ($r = 0.496$; $P = 0.0261$). Next, we determined if abundance and expression patterns of AAV receptor (AAVR) and platelet-derived growth factor receptors (PDGFRa and b), known coreceptors of AAV5, correlated with AAV5 vector transduction. AAVR and PDGFRa expression exhibit a preferential pericentral vein pattern, similar to hepatocellular AAV5 capsid distribution and FVIII expression (**Figure**). There was a significant correlation between FVIII and PDGFRa expression ($r = 0.9546$; $P = 0.0115$) but not PDGFRb ($r = 0.7636$; $P = 0.1330$) or AAVR ($r = 0.1796$; $P = 0.7726$). Levels of DNA repair enzymes that facilitate the transformation of singlestranded vector DNA to circular genomes may also influence AAV vector transduction. The expression of one such exonuclease, Artemis (*DCLER1C*), was significantly correlated with AAV5-FVIII vector DNA levels ($r = 0.6822$; $P = 0.0052$). Next, we identified factors that may contribute to AAV-FVIII transcriptional variability. There was a significant correlation

between FVIII transcripts and mRNA levels of RNF121 (r = 0.7188; P = 0.003), Phf5A (r = 0.5970; P = 0.0154), and
HNF1α (a transcription factor that binds to the promoter of AAV5FVIII-SQ vector; r = 0.7158; P = 0.0054). Overall, we demonstrated that AAV5-FVIII-SQ intersubject variability may be driven by multiple contributing host-mediated mechanisms: transduction, transcription, and protein folding/secretion. Additional studies that further investigate the mechanistic drivers of AAV5 gene therapy variability are ongoing and may help identify predictive biomarkers of transgene expression and/or therapeutic approaches to decrease variability and optimize outcomes.

Figure. AAVR and PDGFRa expression in the liver of C57BL6 male mice shares a pericentral bias with FVIII transgene and vector capsid protein expression



CV, central vein; PV, portal vein.

69. Thermoresponsive Polymer-AAV Nanoparticle Vectors Improved Transgene Expression on Immunized Murine Model

Kai Wang[1], Min Zheng[1], Chengwen Li[2], Zongchao Han[1]
[1]Ophthalmology, The University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Gene Therapy Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC

A great hurdle in adeno-associated virus (AAV) mediated gene therapy is pre-existing or re-administration-related humoral/cellular immune responses. Earlier, we developed an elastin-like polypeptide (ELP)based AAV delivery system and evaluated the performance in vitro in cell cultures. Our results showed that the virions encapsulated in the ELP nanoparticles can be shielded from neutralizing antibodies against AAV capsids. In this study, we have generated more ELP-AAV formulations (ELP $(KV_2F)_{64}$-AAV, ELP V_{60}-AAV, and PNIPAM-AAV) and screened them by neutralizing antibody assay. We found that ELP $(KV_2F)_{64}$ is the most effective polymer to protect AAV virion, and therefore we have used this ELP-AAV vector for further studies. Mass spectroscopy analysis indicated that the polymer was bound to residual K507, R566, and K649 of AAV VP3, and the molecular weight of ELP bonded VP3 was increased by 200 kDa. In vivo studies showed that the ELP-AAV2/9 nanoparticle vectors effectively transduced targeted tissue without tropism change compared to free AAV through subretinal injection, intramuscular injection, and retro-orbital injections. Histological examination indicated that no toxicity was observed on the major organs (kidney, liver, spleen, heart, lung, and brain) of mice injected with ELP-AAV2/9. The ELP-AAV2/9 vectors were also studied on the mice that were immunized with IVIg or prior AAV intramuscular injection via retro-orbital injection 2 hours after IVIg infusion or 2 weeks after intramuscular injection, respectively. Our results suggested that the ELP-AAV2/9 vectors significantly improved the reporter gene expression compared to free AAV and did not change the AAV tropism. Our ongoing studies are focusing on the delivery of using high-doses of ELP-AAV2/9 and alternative AAV serotypes. If successful, this method will create a novel strategy to potentially solve the immunogenic problem in AAV re-administration in clinic.

70. AAV Vector Dose Dependent Redundant and Non-Redundant Roles of TLR9 and IL1R Signaling in CD8⁺ T Cell Activation upon Muscle Gene Transfer

Ning Li, Roland Herzog
Department of Pediatrics, IU School of Medicine, Indianapolis, IN

Adeno-associated viral (AAV) vectors are evaluated in multiple clinical trials for the treatment of neuromuscular disorders. However, immune responses to the transgene product remains a concern. Viral vectors are initially sensed by the innate immune system, which shapes subsequent adaptive immune responses. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), while IL-1 receptors (IL-1Rs) intermediate downstream of pathways trigged by PAMPs or by tissue damage. They act as major sensors of pathogens for innate immune responses. Release of IL-1 cytokine serves as a damage signal to active IL-1R pathways upon infection and/or tissue injury. TLR9 is an endosomal DNA receptor that responds most potently to unmethylated CpG motifs as found in bacterial and viral DNA. Myd88 as a universal and essential adaptor is recruited when TLR9 and IL-1R signaling is activated. Our previous studies found that cross-priming of AAV capsid-specific $CD8^+$ T cells depended on TLR9-MyD88 pathway. Similarly, others documented TLR9dependent $CD8^+$ T cell responses against non-secreted transgene products such as LacZ and hemagglutinin upon muscle-directed AAV gene transfer. In our previous studies, we found that $CD8^+$ T cell responses to a secreted ovalbumin (ova) transgene product were substantially reduced (although not entirely eliminated) upon muscle gene transfer in TLR9-deficient mice [J Innate Immun. 7:302-14]. Here, we performed intramuscular injections with 2 doses of single-stranded ssAAV1-CMV-ova vectors ($2X10^{10}$ and $2X10^{11}$ vg) in wild-type (WT) C57BL/6 and innate sensing knockout (TLR9-/-, MYD88-/- and IL1R-/-) mice. Using MHC tetramer (H2-K^b -SIINFEKL), ova-specific $CD8^+$ T cell frequencies were monitored in peripheral blood for up to 6 weeks. As expected, transgene product-specific $CD8^+$ T cell responses were much reduced in MyD88-/- mice, in which 0.2% and 1.7% $tetramer^+$ of CD8 frequencies were found at low and high doses, respectively. To our surprise, TLR9-/- and IL-1R-/- mice only showed a substantially reduced response (1.2% and 0.1% $tetramer^+$ of CD8) at the low dose when compared to WT animals (12% $tetramer^+$ of CD8, $p<0.0001$, n=5/group), whereas $CD8^+$ T cell responses were similar in TLR9-/- and WT mice (16% and 15% $tetramer^+$ of CD8) and only slightly decrease in IL-1R-/- mice (11% $tetramer^+$ of CD8) at the high dose (n=5/group). To further investigate these findings, we prevented activation of IL-1R by pre-treatment with a combination of IL-1α and IL-1β antibodies followed by IM injections of $2X10^{11}$ vg of ssAAV1CMV-ova vector to TLR9-/- mice. As a result, substantially reduced $CD8^+$ T cell responses (4% $tetramer^+$ of CD8) were observed in these animals compared to TLR9-/- mice that

received isotype antibodies (13% tetramer$^+$ of CD8, p=0.003, n=5/group). Our data reveal that

sensing of the AAV genome by TLR9 and sensing of IL-1 release by IL-1R are both critical for the CD8$^+$ T cell response to the transgene product at lower vector doses. Thus, absence of one of the pathways dramatically impaired the response. However, TLR9 or IL-1R driven signaling pathways are sufficient to drive the response at higher vector doses, so that elimination of one of these now redundant pathways is not sufficient to blunt the response. Rather, both pathways need to be targeted at higher vector doses. We propose that use of CpG-depleted vectors (or TLR9 inhibitors) combined with IL-1R antagonist will be beneficial to prevent CD8+ T cell responses in AAV muscle gene transfer protocols that require high vector doses.

CAR Modified Cellular Therapies

71. Pre-Selected CAR $T_{N/SCM}$ Outperform CAR T_{BULK} in Driving Tumor Eradication in the Absence of Severe CRS and ICANS

Silvia Arcangeli[1], Claudia Mezzanotte[1], Camilla Bove[1], Barbara Camisa[1], Laura Falcone[1], Francesco Manfredi[2], Rossana Norata[3], Francesca Sanvito[4], Maurilio Ponzoni[4], Beatrice Greco[1], Fabio Ciceri[5], Chiara Bonini[2], Attilio Bondanza[1], Monica Casucci[1]

[1]Innovative Immunotherapies, San Raffaele Hospital, Milan, Italy,[2]Experimental Hematology Unit, San Raffaele Hospital, Milan, Italy,[3]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), San Raffaele Hospital, Milan, Italy,[4]Pathology Unit, San Raffaele Hospital, Milan, Italy,[5]Department of Hematology and Stem Cell Transplantation, San Raffaele Hospital, Milan, Italy

Capability of CAR T cells to expand and persist in patients emerged as a fundamental factor accounting for better outcome and durability of antitumor responses. These features inversely correlate with T-cell differentiation, suggesting that the enhanced T-cell fitness typical of early memory T cells may significantly improve CAR T cell therapeutic potential. Presently, however, whether pre-selecting specific memory T-cell subsets before manipulation would be really beneficial is still an open issue, especially as regard toxicity. Therefore, we deeply investigated the efficacy and safety profiles of CAR T cells generated from isolated naive/stem memory T cells ($T_{N/SCM}$), as compared to those derived from unselected T cells (T_{BULK}). As expected, CAR $T_{N/SCM}$ were less lytic than CAR T_{BULK} and produced lower amounts of pro-inflammatory cytokines when stimulated with CD19+ targets *in vitro*, even though displaying a similar proliferative capacity. When challenged against tumor cells in HSPC-humanized mice, limiting doses of CAR $T_{N/SCM}$ showed superior antitumor activity compared to CAR T_{BULK} and the unique ability to protect mice from leukemia rechallenge, together with higher *in vivo* expansion, persistence and

a better CAR T cell fitness. Indeed, as evaluated by BH-SNE algorithm, after leukemia encounter CAR $T_{N/SCM}$ were characterized by prevalence of early memory T-cell subsets, together with the expression of multiple activation markers and a limited enrichment of inhibitory receptors, as opposed to the more exhausted and terminally differentiated phenotype typical of CAR T_{BULK}. Notably, at limiting doses and low tumor burdens no cases of severe Cytokine Release Syndrome (sCRS) were reported. Conversely, when infusing high doses of CAR T cells in mice with

high tumor burdens, sCRS and Immune effector-Cell Associated Neurotoxicity Syndrome (ICANS) were only elicited by CAR T_{BULK}, with more than 50% of mice experiencing a drastic weight loss and increased serum elevation levels of IL-6 and SAA, culminating in the death of the treated mice. Moreover, multifocal brain hemorrhages were only found in the CAR T_{BULK} treated cohort, in contrast to the group infused with CAR $T_{N/SCM}$, in which only one mouse presented with a small hemorrhagic focus. Interestingly, similar results were obtained with CAR $T_{N/SCM}$ harboring a CD28 rather than a 4-1BB co-stimulatory molecule, indicating that CAR $T_{N/SCM}$ are intrinsically less prone than CAR T_{BULK} to trigger detrimental infusional toxicities, independently of the CAR design strategy. In conclusion, $T_{N/SCM}$ pre-selection during CAR T cell manufacturing allows for deeper and more durable antitumor responses in the absence of sCRS and ICANS, significantly widening the therapeutic index of current CAR T cell approaches.

72. CD5 CAR T-Cells Avoid Self-Elimination by Continuously Degrading CD5 Protein

Royce Ma[1], Divya Popat[1], Alex Chaumette[1], Alexandre Carisey[2], Mary K. McKenna[1], Madhuwanti Srinivasan[1], Norihiro Watanabe[1], Malcolm K. Brenner[1], Maksim Mamonkin[1]

[1]Center for Cell & Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Pediatrics, Baylor College of Medicine, Houston, TX

Chimeric antigen receptor (CAR) T-cells for T-cell malignancies have been clinically effective. However, CARs specific to T-cell antigens may also result in extensive fratricide of CAR T-cells, precluding their expansion. This constraint does not apply equally to all the T cell antigens potentially targetable by CAR-Ts. Thus, we have shown that T-cells expressing CD5-specific CARs produce limited initial fratricide and then become resistant to self-targeting despite having high CD5 expression prior to CAR transduction. Such minimal fratricide coincides with rapid and complete loss of detectable CD5 expression on the cell surface without affecting CD5 gene transcription, suggesting post-translational downmodulation of CD5 protein. However, the exact mechanisms underpinning antigen removal and the resulting resistance to fratricide in CD5 CAR T-cells remain unclear; an improved understanding of these processes may guide future efforts to target additional T cell antigens that are currently excluded from consideration due to excessive fratricide. Ligation of CD5 with monoclonal antibodies induces its capping and internalization. Using time-lapse microscopy in T-cells freshly

transduced with CD5 CAR, we observed rapid aggregation and internalization of surface CD5 from the cell membrane. Western Blot analyses revealed complete removal of the CD5 protein in CD5 CAR T-cells thus ruling out epitope masking or intracellular sequestration of the antigen. These results suggested CAR-mediated ligation of CD5 *in cis* triggers continuous internalization and degradation of CD5 protein in T-cells. Indeed, replacing endogenous CD5 protein with an engineered CD5 variant containing myc-/FLAG-tags on the N-/Ctermini resulted in complete loss of both tags in T-cells co-expressing CD5 CAR but not control CD19 CAR indicating the entire CD5 molecule is removed. Neither CAR nor CD5 signaling was required for antigen downmodulation as removal of intracellular signaling portions of each respective molecule did not ablate CD5 downregulation.

The process of CD5 removal can be initiated both *in cis* and *in trans*. CD5 CAR T-cells induced rapid *in trans* downmodulation of surface CD5 expression in both resting and activated primary T-cells upon short coculture with concurrent inter-cellular transfer of CD5 CAR molecules to target T-cells. Importantly, *in trans* removal of CD5 was observed in both normal and malignant T-cell lines suggesting this mechanism can limit availability of CD5 on target T-cells leading to resistance to cytotoxicity. Similarly to the *cis*-mechanism, complete removal of CD5 protein *in trans* was observed in target T-cells expressing dual-tagged [N-]myc/[C-] FLAG CD5 co-cultured with CD5 CAR Ts. However, in contrast to the *cis*-downmodulation, we detected robust release of soluble CD5 protein into culture supernatant during coculture of normal CD5+ T-cells with CD5 CAR T-cells, suggesting CD5 protein can also be shed or secreted from target cells upon contact with CD5 CAR T-cells. This study unravels a novel mechanism of fratricide evasion in T-cells expressing a T lineage antigen-specific CAR mediated by continuous removal of target antigen. Furthermore, rapid downmodulation of CD5 on normal and malignant T-cells may contribute to their resistance to CD5-directed cytolysis. These results are supported by clinical observations from an ongoing Phase I clinical study in which CD5 CAR T-cells could produce complete regression of recalcitrant T-cell tumors without fully ablating the endogenous T-cell compartment. Understanding the mechanisms of fratricide resistance can inform design of other T lineage-specific CARs and improve outcomes in patients with T-cell malignancies.

73. Abstract Withdrawn

74. Investigating the Therapeutic Efficacy of Disruption of Cell Intrinsic Checkpoint Regulator CTLA-4 in Chimeric Antigen Receptor T Cells

Sangya Agarwal[1,2], Angela Aznar[1,2], Tong Da[1,2], Weimin Kong[1,2,3], Mercy Gohil[1,2], Megan M. Davis[1,2], Joseph A. Fraietta[1,2,3], Gabriela Plesa[1,2], Regina M. Young[1,2], David L. Porter[4], Carl H. June[1,2]

[1]Center for Cellular Immunotherapies, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[2]Parker Institute of Cancer Immunotherapy, University of Pennsylvania, Philadelphia, PA,[3]Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[4]Division of Hematology/Oncology, Department of Medicine and Abramson Cancer Center, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Cancer immunotherapy is a rapidly growing field that has led to multiple successful treatment strategies including monoclonal antibodies (MAb), inhibitory receptor (IR) blockade, and adoptive cell transfer (ACT). ACT modified to express chimeric antigen receptors (CARs) can redirect T cells to tumor-associated antigens and has shown impressive clinical efficacy in patients with refractory leukemia and lymphoma. 90% of pediatric patients with acute lymphoblastic leukemia (ALL) respond to CD19 CAR T cell therapy (CART19), whereas only 26-35% of patients with chronic lymphocytic leukemia (CLL) show complete responses (CR). It is unclear why responses are less frequent in CLL compared to ALL. The heavy pre-treatment received by CLL patients likely contributes to lower response rates with T cell therapy, and disease progression is worsened due to profound T cell defects characterized by elevated expression of IRs such as PD-1 and CTLA-4. We hypothesized that disruption of CTLA4 would improve CAR T cell efficacy in CLL based on numerous observations. Fraietta et. al. assessed CLL patient apheresis samples and CART19 products to show that CR was associated with elevated levels of CD27+PD1-CD8+ T memory cells whereas the non-responders (NR) showed an exhausted phenotype with high levels of multiple IRs. We studied 14 patients with advanced, heavily pretreated CLL who received at least one dose of CART19. Patients with CRs exhibited high *in vivo* expansion and persistence of infused CAR T cells, as opposed to NR's. Importantly, at peak levels of *in vivo* CAR expansion, NR's had elevated levels of CTLA-4 expression which correlated with poor CLL patient responses to CART19 therapy. Additionally, multiple studies show CTLA4 is amongst the top 20 differentially upregulated genes in dysfunctional CD8+ T cells from various tumors such as hepatocellular carcinoma, melanoma, non-small cell lung cancer, and pancreatic adenocarcinoma. In summary, these data suggest that eliminating CTLA-4 mediated T cell inhibition can be clinically beneficial. We, therefore, performed disruption of CTLA4 using CRISPR technology in primary human T cells from healthy donors. Our data demonstrate that the knockout (KO) of CTLA-4 leads to maintenance of surface CAR expression and higher tumor clearance in a chronic re-stimulation model using CART19 cells against NALM6 tumor cells. In xenograft models of ALL, KO of CTLA-4 increases the anti-tumor efficacy of CART19 cells. We then performed CTLA4 disruption in CLL patient T cells who had not responded to CAR T cell therapy to determine whether dysfunctional CAR T products from CLL patients can be invigorated by CTLA-4 KO. In the chronic re-stimulation model of NALM6, CTLA-4 KO CAR T cells from CLL patients maintained CAR expression and lower tumor burden, relative to subject-matched unedited CAR T cells. Thus, disruption of CTLA4 in CD19 CAR T cells from NR CLL patient cells endows them with superior anti-tumor efficacy, suggesting that CTLA4 disrupted CAR T cell products may enhance the success rate of CAR T cell therapy for CLL patients. This technology can be feasibly expanded to other tumor indications and

increase the overall efficacy of CAR T cells. These IND-enabling studies will support the translation of this therapy to the clinic.

CAR Modified Cellular Therapies

75. Non-Human Primate Derived CD20 CAR T Cells Elicit a Bystander Effect on CD8 but Not CD4 CAR- T Cells

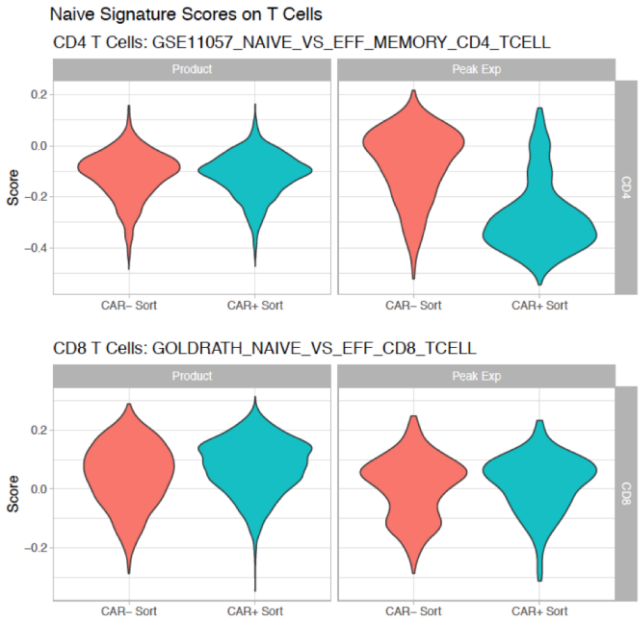
Ulrike Gerdemann[1,2], James Kaminski[3,4], Ryan A. Fleming[3], Emily Ho[5], Victor Tkachev[3], Connor McGuckin[3], Fay Eng[5], Xianliagn Rui[6], Jennifer Lane[3], Michael C. Jensen[7], James Rottman[5], Alex K. Shalek[8], Leslie S. Kean[1,2]

[1]Pediatric Hematology/Oncology, Dana Farber Cancer Institute, Boston, MA,[2]Pediatric Hematoloty/Oncology, Boston Children's Hospital, Boston, MA,[3]Pediatric Hematology/Oncology, Boston Children's Hospital, Boston, MA,[4]Broad Institute of MIT and Harvard, Boston, MA,[5]BluebirdBio, Cambridge, MA,[6]Pediatric Hematology/Oncology, Boston Children' Hospital, Boston, MA,[7]Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle, WA,[8]Broad Institute of MIT and Harvard, Cambridge, MA

The recent clinical successes of CAR T cell (CAR T) therapy are reshaping the field of hematology-oncology. However, despite the significant response to CD19 CAR T therapy in B-ALL, 10-20% of patients fail to enter remission and, perhaps more importantly, 30-50% of patients who achieve remission relapse within a year. Given these results, it is critical to understand not only the drivers of CAR T effector functions but also the mechanisms by which one can induce a tumoricidal bystander effect in surrounding effector cells. Performing detailed analysis of CD19 CAR Ts in human subjects is challenging mostly due to the limited sample volume available. To address this shortcoming, we have now utilized a clinically relevant Non-Human Primate (NHP) CD20 CAR T model to identify mechanisms of the CAR T specific immune response. For this we adoptively transferred CD20 CAR Ts to lymphodepleted rhesus macaques. CAR T expansion resulted in B cell ablation, associated with clinical symptoms of cytokine release syndrome. CAR T persisted an average of 4 weeks, at which point loss of CAR T was followed by B cell recovery. Flow analysis of the CAR- T population showed only minimal activation status of CD4 CAR- Ts and expansion of CAR Ts was more prominent in CD8 T cells. To further reveal the bystander effect of CAR- Ts we performed single cell sequencing analysis of sorted CAR+ and CAR- Ts from the product and at time of maximum proliferation. As expected, CAR- and CAR+ Ts in the infusion product showed similar transcriptomic profiles, however, state of activation differed significantly between CD4 CAR- and CD4 CAR+ Ts at peak of expansion (Wilcoxon test, p-value <0.001). While after infusion CD4 CAR- Ts reverted to memory- like state, CD4 CAR+ Ts maintained an effector state. In contrast, CD8 CAR+ and CD8 CAR- Ts both displayed a very similar effector pattern in the product and at maximum expansion, suggesting, that CAR- CD8 Ts, in contrast to CAR- CD4 Ts, are subject to a significant bystander effect. This is also reflected in the analysis of differentially expressed genes: at time of peak expansion we detected > 200 differentially expressed genes in between CD4 CAR- and (n=832) CAR+ Ts (n=594). In contrast differential gene expression analysis of CD8 CAR+ (n=163) vs CAR- Ts (n=556) at peak expansion did not demonstrate significant differences. Ingenuity Pathway Analysis comparing differentially expressed genes in CD8 CAR+ and CAR- Ts in peak expansion vs infusion product suggests a role of mir155-

CAR Modified Cellular Therapies

driven pathways in the CD8 CAR- T bystander effect.In summary these data show for the first time that a CAR induced bystander effect is predominantly elicited in CD8 CAR- Ts. Further dataset analyses as TCR based T cell tracking are underway to uncover additional mechanism of the immune regulation of CAR Ts in the NHP model.



The signature score for each cell was calculated using the software package VISION, which treats cell signatures from MSigDB as scores (Upregulated = +1,Downregulated = -1), and multiplies these scores by the normalized expression counts in each cell. Results are displayed as violin plots, separated by CAR+/CAR- status, and timepoint.

76. Enhanced Generation of T-Cell Derived Naïve Pluripotent Cells as a Renewable Cell Source for the Mass Manufacture of Off-the-Shelf CAR T Cell Therapies

Yi-Shin Lai, Hui-Ting Hsu, Mochtar Pribadi, Greg Bonello, Megan Robinson, Pei-Fang Tsai, Bi-Huei Yang,
Gloria Hsia, Amanda Yzaguirre, Raedun Clarke, Tom Lee, Ramzey Abujarour, Bahram Valamehr

Fate Therapeutics, San Diego, CA

Induced pluripotent stem cells (iPSCs) are a promising renewable cell source for the mass manufacture of uniform chimeric antigen receptor (CAR) T cell products that can be banked and validated in advance to relieve major manufacturing cost and logistical obstacles. iPSCs can be generated from a variety of somatic cell types (commonly fibroblasts or CD34+ cells) but T cells are an ideal starting material for the derivation and engineering applications

related to iPSC-derived T cell therapies. However, a major obstacle toward the use of T cells for the generation of iPSCs is that specialized cells such as T or B lymphocytes are not readily amenable to cellular reprogramming and often fail to dedifferentiate into bona fide pluripotent cells. To develop a robust non-integrating plasmid-based system for T cell reprograming, we applied both genomics and functional screening approaches to identify novel reprogramming factors that could markedly improve reprogramming of terminally differentiated somatic cells, including T cells. To enhance cellular reprogramming and induce naïve pluripotency, we used our previously described stage-specific media supplemented with small molecule drivers of naïve pluripotency and inhibitors of differentiation (MEKi, GSK3i, ROCKi and TGFbi). Using the new set of reprogramming factors that includes the master regulator of pluripotency gene *OCT4* and optimized medium formulations, iPSCs were generated in a highly efficient manner from T cells derived from multiple donors, with the fraction of TiPSCs reaching 7-15% of the population by day 18 post induction of cellular reprogramming and further increasing to 36-50% at day 26, enabling the ability for multiplexed engineering at the iPSC stage. To generate clonal TiPSC clones, we sorted single reprogrammed cells into 96-well plates and expanded multiple TiPSC clones for extensive characterization and selection. Quantitative PCR analysis revealed that all TiPSC clones were free of reprogramming plasmids and no longer had the potential to dedifferentiate. RNA sequencing data confirmed that the TiPSC clones were equivalent to control iPSC lines and pluripotency was further confirmed by the ability of the TiPSC clones to differentiate into representatives of the 3 germ layers in a trilineage differentiation assay. The generated feeder-free and single cell-derived TiPSCs showed enhanced expression of naïve pluripotency markers (e.g. KLF4, DMNT3L, PRDM14) and lower expression of primed pluripotency markers (e.g. THY1, OTX2, ZIC2). Importantly, TiPSC clones differentiated efficiently into homogenous T cell populations and maintained normal karyotype over extended culture duration. Next, we tested if the generated TiPSC clones maintained stable pluripotent profile and genomic integrity following stress-inducing manipulations including multiplexed engineering, subcloning and cryopreservation. Selected cryopreserved TiPSC clones were thawed and engineered by CRISPR-mediated insertion of CAR into the TCR alpha constant locus, re-cloned at the single cell level and re-banked. The engineered CAR+ TiPSC clones continued to maintain a homogenous pluripotent phenotype and normal karyotype, and differentiated into CAR T cells that exerted effective cytotoxicity against target cancer cells. Collectively, our data describe improvements in the generation of footprint-free and single cell-derived naive TiPSCs that are amenable for multiple rounds of multiplexed engineering, subcloning and cryopreservation and facilitate the replacement of donor-derived T cells as the preferred source for a more consistent, homogenous, cost effective and off-the-shelf CAR T cell product.

77. Precise Targeting of AML with First-inClass OR / NOT Logic-Gated Gene Circuits in CAR-NK Cells

Brian S. Garrison[1], Han Deng[1], Gozde Yucel[1], Nicholas W. Frankel[1], Marcela Ayala Guzman[1], Russell Gordley[1], Michelle Hung[1], Derrick Lee[1], Marcus Gainer[1], Kathryn Loving[1], Jenny Chien[1], Tiffany Pan[1], Wesley Gorman[1], Travis Wood[1], Wilson Wong[2], Philip Lee[1], Tim Lu[1], Gary Lee[1]

Background: Given the poor prognosis and long-term survival of relapsed/refractory acute myeloid leukemia (AML) patients, more efficacious therapies are greatly needed. While chimeric antigen receptor (CAR) cell therapies have provided some extraordinary clinical responses, developing effective CAR cell therapies for AML has been challenging due to: (a) the lack of a single target antigen robustly expressed across both AML leukemic stem cell (LSC) and immature leukemic blast cell subpopulations, and (b) the lack of truly AML-specific target antigens, since current targets are also expressed on healthy tissues and may result in off-tumor toxicity. Using logic-gated gene circuits, we are engineering CAR-NK cells to overcome these longstanding challenges. **Methods:** To maximize clearance of AML tumor cells and minimize toxicities, we used a proprietary bioinformatics pipeline to identify the optimal combinations of AML and healthy tissue target antigens to incorporate into OR and NOT logic-gated CAR gene circuits. These first-in-class CD33 OR FLT3 NOT Endomucin (EMCN) gene circuits enable allogeneic CAR-NK cells to target CD33 and/or FLT3 expressing AML tumor cells (LSCs and blasts) but not healthy FLT3+ hematopoietic stem cells (HSCs). **Results:** First, for the CD33 OR FLT3 activating CAR (aCAR) portion of the logic circuit, we demonstrated that engineered primary human NK cells expressing both CD33 and FLT3 aCARs exhibited up to 75% cytotoxicity and significant cytokine secretion (GrB, IFN-g, and TNF-a) against multiple leukemia cell lines in vitro, including MOLM13, THP1, and SEM. Importantly, these CD33 OR FLT3 CAR-NK cells also significantly reduced tumor burden and improved mouse survival within a MOLM13 xenograft leukemia model. Second, for the NOT gate portion of the logic circuit to protect FLT3+ healthy HSCs, we developed inhibitory CARs (iCARs) (NOT gates) that recognize the HSC-specific EMCN surface antigen, which is expressed on up to 70% of healthy HSCs but not AML cells. We demonstrated that FLT3 aCAR-NK cells engineered with an EMCN-specific iCAR protected over 50% of FLT3+ EMCN+ cells from FLT3 aCAR-mediated cytotoxicity. Next, to more closely replicate a clinical context, we mixed FLT3+ EMCN- (AML-like) and FLT3+ EMCN+ (HSC-like) target cells and demonstrated that FLT3 NOT EMCN CAR-NK cells exhibit preferential killing of FLT3+ EMCN- target cells, demonstrating that our NOT-logic gene circuit controls NK-mediated responses on a cell-by-cell basis. **Conclusion:** This work represents the first time NK cells have been engineered with OR and NOT logic-gated CAR gene circuits, wherein the OR gate provides increased AML

[1] Senti Biosciences, South San Francisco, CA,[2]Department of Biomedical Engineering, Boston University, Boston, MA

LSC/blast tumor clearance (to prevent relapse), and the NOT gate protects healthy HSCs from offtumor toxicity, which may preserve hematopoiesis and mitigate the need for bone marrow transplant. Beyond AML, logic-gated CAR-NK cell technology has applicability to other cancer-associated antigens limited by potential off-tumor toxicity.

Gene Therapies for Hemoglobinopathies

78. Early Results from a Phase 1/2 Study of ARU-1801 Gene Therapy for Sickle Cell Disease (SCD): Safety and Efficacy of a Modified Gamma Globin Lentivirus Vector and Reduced Intensity Conditioning Transplant

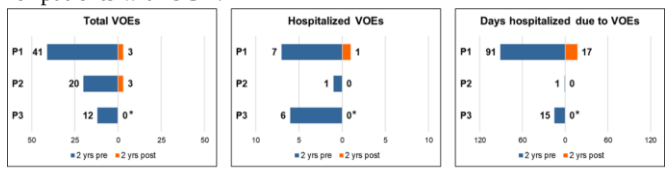
Michael Grimley[1], Monika Asnani[2], Archana Shrestha[1], Sydney Felker[1], Carolyn Lutzko[1], Paritha I. Arumugam[1], Scott Witting[1], Jennifer Knight-Madden[2], Omar Niss[1], Charles T. Quinn[1], Christopher Lo[3], Courtney R. Little[3], Joseph W. McIntosh[3], Punam Malik[1]

[1]Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[2]Carribean Institute for Health Research, Kingston, Jamaica,[3]Aruvant Sciences, New York, NY

ARU-1801 is an investigational gene therapy drug product to treat sickle cell disease, consisting of autologous CD34+ hematopoietic stem cells and progenitors (HSPCs) transduced with a lentiviral vector (LV) encoding a modified γ-globin^{G16D} gene. It is being evaluated in patients with SCD in the ongoing Phase 1/2 MOMENTUM study (NCT02186418). Preliminary studies in SCD mice have suggested HbF^{G16D} may have a more potent anti-sickling effect than wild-type HbF. As a high potency anti-sickling globin, HbF^{G16D} is believed to allow ARU-1801 to be effective with reduced intensity conditioning (RIC), resulting in fewer toxicities and lower resource utilization than myeloablative approaches, expanding access to gene therapy to a broader group of SCD patients. Here, we present long-term clinical data on the first two patients (P1 and P2) and 10-month follow-up on patient 3 (P3), the first patient treated with a newer manufacturing process (Process II) to improve engraftment and increase HbF^{G16D} expression. As of December 2021, 3 patients treated with ARU-1801 have follow-up of >9 months. ARU-1801 demonstrated a favorable safety profile with no treatment-related adverse events to date. RIC resulted in neutrophil engraftment within 7-9 days (median 7 days), and platelet engraftment within 6-12 days (median 7 days). Under the initial manufacturing process, P1 has shown steady VCN of 0.2, stable expression of 20% HbF^{G16D} and 31% total anti-sickling globin (ASG, composed of endogenous HbF, HbA2 and ARU-1801-derived HbF^{G16D}), with 64% F-cells at 2 years post ARU-1801 infusion. P2 had a sub-therapeutic exposure to melphalan secondary to renal hyperfiltration and rapid clearance of melphalan, resulting in lower engraftment. Despite lower engraftment (VCN of 0.1) and hence lower HbF^{G16D} levels, P2 maintained stable 22% ASG expression and

36% F-cells at 2 years post ARU-1801 infusion due to sustained increases in endogenous HbF and HbA2. Under the new manufacturing process, P3 has demonstrated a stable VCN of 0.7 (latest measurement at month 9) with 41% ASG expression and 27% HbF^{G16D} at month 10. Furthermore, P3 has 92% F-reticulocytes at month 6, showing near pan-cellular expression of HbF. Treatment with ARU-1801 has resulted in remarkable improvement in clinical outcomes. As shown in Figure 1, patients had 12-41 VOEs (median, 21) in the 24 months prior to treatment with ARU-1801 and were hospitalized for 1-7 of those VOEs (median, 6). Through 24 months post ARU-1801 treatment, Patients 1 and 2 have seen 93% and 85% reductions in the number of VOEs, and Patient 3 has had no VOEs through 10 months of follow-up, a 100% reduction. The corresponding

cumulative days in hospital associated with those VOEs has decreased from 1-91 days (median, 15) to 0-17 (median, 0), representing an average 93.8% reduction. These results are an encouraging sign of the therapeutic benefit of ARU-1801 with RIC for patients with SCD.



79. Immunereconstitution in Transfusion Dependent Beta-Thalassemia Patients Treated with Hematopoietic Stem Cell Gene Therapy

S. Scaramuzza[1], S. Marktel[1,2], F. Giglio[2], M. P. Cicalese[1,3], M. R. Lidonnici[1], C. Rossi[1], V. Calbi[1,3], N. Masera[4], E. D'Angelo[5], N. Mirra[5], R. Origa[6], I. Tartaglione[7], S. Perrotta[7], G. Viarengo[8], L. Santoleri[9], R. Milani[9], S. Gattillo[9], A. Calabria[1], E. Montini[1], G. Graziadei[10], L. Naldini[1,11], M. D. Cappellini[10], A. Aiuti[1,3,11], F. Ciceri[1,2,11], G. Ferrari[1,11]

[1]SR-TIGET, Milano, Italy,[2]Haematology and BMT Unit, IRCCS San Raffaele Scientific Institute, Milano, Italy,[3]Pediatric Immunohematology, IRCCS San Raffaele Scientific Institute, Milano, Italy,[4]Pediatric Department University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy,[5]Pediatric Clinic/ DH, Fondazione IRCCS Ca' Granda, Milano, Italy,[6]University of Cagliari, Cagliari, Italy,[7]Università degli studi della Campania "Luigi Vanvitelli", Napoli, Italy,[8]Immunohematology and Transfusion Medicine Service, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy,[9]Blood Transfusion Service, IRCCS San Raffaele Scientific Institute, Milano, Italy,[10]Rare Disease Center, Fondazione IRCCS Ca' Granda, Milano, Italy,[11]University Vita-Salute San Raffaele, Milano, Italy

Transfusion-dependent β-thalassemia (TDT) is a disorder due to mutations in the gene encoding the ß-globin chain causing a reduced or absent production of hemoglobin A leading to severe anemia and lifelong transfusion dependence.Gene therapy has been recently accepted as a possible alternative to the only curative treatment

represented by allogeneic bone marrow (BM) transplantation. We developed a gene therapy approach based on autologous mobilized hematopoietic stem cell transduced by LV vector, expressing human ß-globin gene, administered by intrabone injection, following a myeloablative conditioning (NCT02453477). Nine patients with severe TDT with different genotypes have been treated with a drug product with a median cell dose of $19.5x10^6$ $CD34^+$ cells/kg, a transduction efficiency from 38 to 77% and a median vector copy number/genome (VCN) in bulk $CD34^+$ cells of 0.9 (range 0.7-1.5). Overall, gene therapy was generally well-tolerated with no adverse events related to the investigational product. No severe infectious-related adverse events were reported, except for those related to neutropenia as expected after conditioning. Insertion site analysis demonstrated highly polyclonal engraftment with no clonal dominance. Clinical outcome showed a reduction of transfusion requirement both in frequency and volume in adult patients up to more than 50%. Among the pediatric patients, 4 out of 6 discontinued transfusions shortly after gene therapy and are transfusion-independent at the last follow-up (up to 60 months). A robust and persistent engraftment was observed in 7 out of 9 patients, with a marking of BM progenitors that, in engrafted patients, ranged between 25.3 and 79.8% and with a median VCN in $CD34^+$ cells of 0.53 (range 0.34-2.21). As a relevant target, BM erythroid cells were stably marked (VCN range 0.3-2.5).Similar values were retrieved in the myeloid compartment and B lymphocytes while a lower VCN (range 0.12-1.48) was observed in $CD3^+$ cells. Focusing on lymphocytes, different rates of cell count increase were reported in B and T cells. In particular, $CD4^+$ T cells remained below the normal range for a prolonged period in all the patients, resulting in an inverted CD4/ CD8 ratio, consistent with reported allogeneic bone marrow transplant experience. Flow-cytometry, TREC and KREC evaluation performed at different time-points showing a slow but progressive recovery of immunocompetency. Importantly, despite a slow increase in the number, both innate and adaptive immune cells showed good response to mitogens and pathogens with an adequate antibody titer documented in response to vaccinations. All patients were enrolled in a long-term follow-up study that will provide results on long-term clinical efficacy and safety of this gene therapy.

80. Multiplex Base Editing of Hematopoietic Stem and Progenitor Cells to Enrich Therapeutic Cells Post Engraftment

Olivier Humbert[1], Emily S. Fields[1], Mallory J. Llewellyn[1], Savannah M. Cook[1], Gregory A. Newby[2,3], Jonathan Yen[4], George S. Laszlo[1], Stefan Radtke[1], Roland B. Walter[1,5], Mitch J. Weiss[4], David R. Liu[2,3], Hans-Peter Kiem[1,5]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA,[2]The Broad Institute of Harvard and MIT, Cambridge, MA,[3]Harvard University, Cambridge, MA,[4]St. Jude Children Research Hospital, Memphis, TN,[5]University of Washington, Seattle, WA A significant obstacle in current hematopoietic stem cell (HSC) gene therapy studies is the inability to consistently achieve sufficiently high engraftment of engineered cells to provide long-term therapeutic efficacy. Here we propose a strategy to increase engraftment of genome-edited cells after transplantation using multiplex base editing. Base editors are ideal for multiplexing since they introduce precise genetic alterations without double-stranded DNA breaks and thus prevent risk for chromosomal translocations. CD34+ cells were simultaneously edited both at a therapeutic gene and at a selection gene for evaluation in transplantation studies. As therapeutic target, we focused on the gamma-globin (HBG) locus for the reactivation of fetal hemoglobin (HbF) to treat hemoglobinopathies. This target has already been validated using CRISPR/Cas9-based approaches in several pre-clinical studies, including by our group. As edit that can be enriched by selection, we investigated the myeloid differentiation antigen CD33. Since CD33 is widely expressed on neoplastic myeloid cells, a wide array of CD33-directed drugs has been developed and tested. Previous findings demonstrated that inactivation of CD33 in HSCs by CRISPR/Cas9-editing does not impact the engraftment and multilineage differentiation potential of these cells. We used the adenine base editor ABE8e as editing platform, which was recently evolved from ABE7.10 for more robust activity. Human CD34+ cells electroporated with ABE8e mRNA targeting both the HBG and CD33 sites were transplanted in the murine model. Editing efficiency in infused cells averaged 40% for HBG and 80% for CD33. Notably, clonal assays demonstrated that about 75% of treated cells displayed edits at both targets in the same cell. Engraftment was comparable between the multiplex-edited group and the mock electroporated group. Furthermore, editing levels in peripheral blood (PB) remained high for both targets during the course of the experiment. Monocytes generated from engrafted cells displayed considerably reduced CD33 expression. Treatment of the animals with gemtuzumab ozogamicin confirmed resistance of edited cells to this highly potent CD33 antibody-drug conjugate. To further validate our multiplex editing strategy in an autologous transplantation setting, we utilized our nonhuman primate model described previously (Humbert et al., STM 2019). The CD34+CD90+ HSC-enriched cell population was edited with the same ABE8e-based approach as described above, yielding editing efficiencies in infused cells averaging 50% and 70% for HBG and CD33, respectively, with 70% containing edits at both targets. At 3 months post-transplantation, editing measured in total nucleated cells stabilized at about 10% (HBG) and 20% (CD33). CD33 expression measured in PB granulocyte was reduced to 65% as compared to >90% in a control animal, and HbF expression, as determined by F-cell frequency, reached 12% of total red blood cells. We will next use CD33-directed drugs in this animal to determine the in vivo ability to efficiently enrich for CD33- and thus HBG-edited cells as a means to enhance HbF to therapeutic levels for hemoglobinopathies. Together, our results demonstrate efficient engraftment and persistence of multiplex base edited CD34+ or CD34+CD90+ HSC-enriched cells in murine and NHP models. If enrichment is successful, this approach could serve as the basis for a broadly applicable method to treat other genetic blood diseases.

81. In Vivo HSC Gene Therapy for Hemoglobinopathies: A Proof of Concept Evaluation in Rhesus Macaques

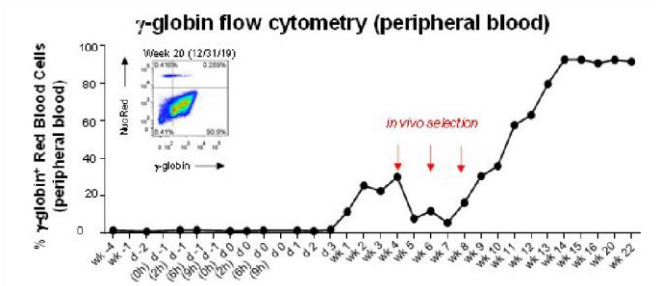
Chang Li[1], Hongjie Wang[1], Sucheol Gil[1], Aphrodite Georgakopoulou[1], Stefan Radtke[2], Evangelia Yannaki[3], Thalia Papayannopoulou[4], Hans-Peter Kiem[5], Andre Lieber[1]

[1]Division of Medical Genetics, University of Washington, Seattle, WA,[2]Fred Hutchinson Cancer Research Center, Seattle, WA,[3]G. Papanicolaou Hospital, Thessaloniki, Greece,[4]Division of Hematology, University of Washington, Seattle,
WA,[5]Division of Medical Genetics, Fred Hutchinson Cancer Research Center, Seattle, WA

Current gene therapy or genome editing studies for hemoglobinopathies require highly sophisticated medical facilities to perform hematopoietic stem cell (HSC) collections/selections and genetic modifications. In addition, patients receive high-dose chemotherapy to facilitate engraftment of gene-modified cells. Thus, current gene therapy protocols will not be accessible to most patients suffering from hemoglobinopathies. Here we describe a highly portable and scalable approach using in vivo HSC gene therapy to potentially overcome these limitations. The central idea of our in vivo HSC gene therapy approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus HDAd5/35++ gene transfer vector system. Transgene integration is

either achieved by a Sleeping Beauty transposase (SB100x) in a random pattern or by homology-directed-repair into a safe genomic harbor site. Currently, an in vivo selection system (involving the mgmt^{P140K} gene/low-dose O^{6}BG/BCNU) is employed to achieve >80% marking levels in peripheral blood cells. We demonstrated safety and efficacy of our approach in mouse models for thalassemia intermedia, Sickle Cell Disease, and hemophilia A, where we achieved a phenotypic correction. We now present data in 3 rhesus macaques. We show that treatment with G-CSF/AMD3100 resulted in efficient HSC mobilization into the blood circulation and subsequent intravenous injection of the HDAd5/35++ vector system (total 1-3 x10^{12} vp/kg, in two doses) was well tolerated. The longest follow-up thus far is 24 weeks after in vivo HSC transduction with a human-γ-globin expressing HDAd5/35++ vector. After in vivo selection with O^{6}BG plus low dose of BCNU, γ-globin marking in peripheral red blood cells rose to ~90% and was stable for the duration of the study (see Figure). γ-globin levels in red blood cells were ~18% of adult α1-globin (by HPLC). No abnormalities in genome and transcriptome analyses of animal #1 were found at the time of scheduled necropsy. We show that a new prophylaxis regimen (dexamethasone, IL-6R, IL-1bR antagonists, saline bolus IV) was able to mitigate all side effects associated with intravenous HDAd5/35++ vector administration. Analysis of day 3 bone marrow showed 30% transduced HSCs. Vector DNA biodistribution studies demonstrated very low or absent transduction of most tissues (including testes and CNS). Analysis of bone marrow showed efficient, preferential HSC transduction and re-homing of transduced CD34$^+$CD90$^+$ cells to the bone marrow. At week 4, about 5% of progenitor colony-forming cells demonstrated stable transduction with integrated vector, and this frequency increased after starting the in vivo selection. The level of human mgmt^{P140K} mRNA expression in PBMCs also increased after in vivo selection. This is the first proof-of-concept study that in vivo HSC gene therapy could be feasible in humans without the need of high-dose chemotherapy

conditioning and without the need for highly specialized medical facilities.



82. Hematopoietic Reconstitution and Lineage Commitment in HSC Gene Therapy Patients Are Influenced by the Disease Background

Andrea Calabria[1], Giulio Spinozzi[1], Fabrizio Benedicenti[1], Daniela Cesana[1], Luca Del Core[1,2], Serena Scala[1], Samantha Scaramuzza[1], Maria Rosa Lidonnici[1],
Alessandra Albertini[1], Simona Esposito[1], Fabiola De Mattia[1], Maryam Omrani[1], Valeria Calbi[1], Francesca Fumagalli[1], Marco Grzegorczyk[2], Ernst Wit[3], Giuliana Ferrari[1,4], Luigi Naldini[1,4], Alessandro Aiuti[1,4], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Bernoulli Institute for Mathematics, Groningen, Netherlands,[3]Universita della Svizzera Italiana, Lugano, Switzerland,[4]Vita Salute San Raffaele University, Milan, Italy

Lentiviral vector (LV) based hematopoietic stem cell (HSC) gene therapy (GT) applications have shown a favorable efficacy and safety profile for the treatment for a variety of genetic diseases. While this is a promising strategy for the treatment of diverse diseases, several factors, including transplantation protocols, patients' age and the underlying genetic disease, may impact on the kinetics of hematopoietic reconstitution, lineage specification, efficacy, and safety. To understand the impact of patient-specific factors and disease background on the hematopoiesis after transplantation, we studied the clonal reconstitution and multilineage potential over time in 48 HSC-GT patients affected by 3 different diseases: 29 with metachromatic leukodystrophy (MLD, a neurodegenerative lysosomal storage disorder), 10 with WiskottAldrich syndrome (WAS, a B and T cell immunodeficiency) and 9 with β-thalassemia (β-Thal, a hemoglobinopathy). As reported previously, these HSC-GT treatments resulted in clinical benefits for most patients. We analyzed the LV genomic Integration Sites (IS), a genetic marker for clonal identity, from the DNA of CD34$^+$ cells as well as myeloid, B, T and erythroid cell lineages purified from blood and bone marrow, harvested at different time points after HSC-GT (longest follow up to 9 years). Integration site analysis yielded >3 million unique IS and showed highly polyclonal reconstitution, multilineage marking and no signs of genotoxicity in all patients.Regardless of disease background, all patients showed a similar pattern of hematopoietic reconstitution over time,

characterized by: an early phase up to 9 months after GT, where the myeloid cells are highly polyclonal whilst T and B cells have a less complex repertoire; a second phase of 9 up to 18 months, where the polyclonality of lymphoid cells increases; and a third phase where the complexity of the lineages decreases and stabilizes. During the early phase of hematopoietic reconstitution an average of 80,000 active hematopoietic stem and progenitor cell (HSPCs)/patient contributed to hematopoiesis. However, the active HSPC pool decreased to an average 11,000 after 9 months, suggesting that short lived progenitors have a relevant role in sustaining the early phases of hematopoietic reconstitution. Differences across diseases were found when we analyzed the multilineage potential of individual clones and their commitment towards a specific lineage over time. In MLD patients multilineage clones reached a proportion of 75% on the total engineered clones, decreased over time to 50%, remaining stable thereafter. Myeloid-committed clones increased over time and stabilized at 40%. The decrease in multilineage clones over time was also observed in WAS and β-Thal patients. However, in WAS patients we did not observe a concomitant increase in myeloid committed cells rather an increase of the commitment in the T-cell lineage, as expected by the selective advantage in T cells, whereas in β-Thal patients several clones showed erythroid commitment.These novel data suggest that the disease condition influences the proportion and the type of lineagecommitted cells over long periods of time, and that the engrafted HSPC pool respond to the disease-specific physiopathology in a dynamic fashion to restore normal hematopoiesis.

83. Lentiviral Mediated Gene Therapy for Pyruvate Kinase Deficiency: Updated Results of a Global Phase 1 Study for Adult and Pediatric Patients

José Luis López Lorenzo[1,2], Ami J. Shah[3,4,5], Susana Navarro[2,6,7], Julián Sevilla[8], Lucía Llanos[1,2], Begoña Pérez
Camino de Gaisse[1,2], Sol Sanchez[1,2], Bertil Glader[5,9], May Chien[5,9], Oscar Quintana-Bustamante[2,6,7], Brian C. Beard[10], Kenneth M. Law[10], Miriam Zeini[10], Grace Choi[10], Eileen Nicoletti[10], Gayatri R. Rao[10], Maria Grazia Roncarolo[3,4,5], Juan A. Bueren[2,6,7], Jonathan D. Schwartz[10], José C. Segovia[2,6,7]

[1]Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain,[2]Unidad Mixta de

Terapias Avanzadas, Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid, Spain,[3]Center for Definitive and Curative Medicine, Stanford University, Stanford, CA,[4]Div. of Pediatric Hematology/Oncology/Stem Cell Transplant and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA,[5]Stanford Children's Hospital, Palo Alto, CA,[6]Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain,[7]Instituto de Innovación Biomédica, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain,[8]Hospital Infantil Universitario Niño Jesus, Madrid, Spain,[9]Dept. of Pediatrics, Div. of Hematology/Oncology, Stanford University School of Medicine, Stanford, CA,[10]Rocket Pharmaceuticals, Inc., Cranbury, NJ

Introduction: Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia that results from mutations in the *PKLR* gene leading to decreased red cell pyruvate kinase activity and impaired erythrocyte metabolism. Manifestations include anemia, reticulocytosis, splenomegaly and iron overload, and may be lifethreatening in severely affected individuals. Current treatments are limited to blood transfusions, iron chelation therapy, and splenectomy which are associated with significant side effects. Preclinical studies in a clinically relevant PKD murine model have demonstrated that infusion of gene-modified Lin^- bone marrow (BM) cells may ameliorate the PKD phenotype. Based on compelling preclinical data, a global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate the feasibility and safety of lentiviral mediated gene therapy in adults and pediatric subjects with severe PKD. **Methods:** A total of 6 adult and pediatric subjects with severe PKD (defined as severe and/or transfusion-dependent anemia despite prior splenectomy) will be enrolled. Peripheral blood (PB) hematopoietic stem cells (HSCs) are collected on 2 consecutive days via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and plerixafor. $CD34^+$ HSCs are enriched, transduced with PGK-coRPKWPRE lentiviral vector, and cryopreserved. Following release testing of the investigational product (IP), RP-L301, myeloablative conditioning with therapeutic drug monitoring guided busulfan is administered over 4 days. RP-L301 is then thawed and infused. Patients are followed for safety and efficacy assessments for 2 years post-infusion. **Results:** As of January 2021, 2 adult subjects have been treated. Patient 1 received $3.9x10^6$ $CD34^+$ cells/kg with mean vector copy number (VCN) of 2.73. At 3 months post-infusion hemoglobin has normalized, with PB VCN of 1.55. Patient 2 received $2.4x10^6$ $CD34^+$ cells/kg with mean VCN of 2.08. Neutrophil engraftment occurred within 2 weeks for both patients. No adverse events have been attributed to RP-L301. 6- and 3-month post-treatment data will be presented for Patients 1 and 2, respectively. **Conclusions:** Hematopoietic stem cell mobilization using G-CSF and plerixafor appears feasible and effective in adult PKD patients. RP-L301 was successfully manufactured to meet the required specifications for the Phase 1 clinical study and administered without short-term infusion related complications. Preliminary efficacy was evident in Patient 1 during the initial 3 months post-RP-L301 as demonstrated by hemoglobin normalization and PB genetic markings.

84. Base Editing of the -200 Region of the γ-globin Promoters Leads to Fetal Hb Reactivation and Rescues the Sickle Cell Disease Phenotype in Primary Patient Cells

Panagiotis Antoniou[1], Giulia Hardouin[1], Pierre Martinucci[1], Giacomo Frati[1], Megane Brusson[1], Marion Rosello[2], Giulia Maule[3], Filippo Del Bene[2], Anna Cereseto[3], Wassim El Nemer[4], Jean-Paul Concordet[5], Annarita Miccio[1]

[1]IMAGINE Institute, Paris, France,[2]Institut de la Vision, Paris, France,[3]CIBIO, Trento, Italy,[4]Institut National de la Transfusion Sanguine, Paris, France,[5]Museum National d'Histoire Naturelle, Paris, France

β-hemoglobinopathies, β-thalassemia and sickle cell disease (SCD), are caused by mutations affecting the production of the adult hemoglobin (Hb). Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an

attractive therapeutic option. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing hereditary persistence of fetal Hb (HPFH) in adult life. To reactivate fetal γ-globin expression, nuclease-based editing approaches have been explored. Site-specific nucleases, however, generate double-strand breaks (DSBs) in the genome, raising safety concerns for clinical applications. Base editing (BE) allows the introduction of point mutations (C>T by cytidine base editors, CBEs; A>G by adenine base editors or ABEs) without generating DSBs.HPFH mutations in the promoters of the 2 γ-globin (*HBG1/2*) genes either disrupt the binding sites (BS) of fetal Hb (HbF) repressors or generate BS for HbF activators. In particular, mutations clustering ~200 nucleotides upstream of the *HBG* TSSs either reduce LRF binding (e.g. -197 C>T) or recruit KLF1 (-198 T>C). In this study, we used base editors to recapitulate HPFH mutations in the -200 region in the *HBG* promoters.First, we explored the BE system to introduce C>T mutations in the LRF BS. The absence of the canonical *Spy*Cas9 NGG PAM close to the LRF BS prompted us to test a variety of non-NGG CBEs including CBE-NRCH, CBE-SpG, CBE-SpRY and a novel BE containing NAA PAM Cas9 variant. CBEs edited 7 out of 8

cytidines of the LRF BS in an erythroid cell line (K562) with efficiencies of up to ~60%. These C>T conversions include not only known HPFH mutations but also HPFH-like mutations that can further impair LRF binding.We tested this strategy in HSPCs from SCD patients and achieved BE efficiencies of up to ~45%. A progenitor assay indicated no alteration in the multilineage differentiation of edited HSPCs. HSPCs were differentiated in mature red blood cells (RBCs). The expression of erythroid markers was similar in control and edited samples and the production of mature RBCs was not affected by the BE treatment. We observed a potent γ-globin reactivation with a high frequency of HbF^+ cells and a concomitant decrease in the HbS content/cell, as detected by RT-qPCR, HPLC and flow cytometry. Importantly, the pathological RBC sickling phenotype was substantially improved in the edited samples. Finally, we used GUIDE-seq coupled with targeted sequencing to evaluate the potential off-target activity of gRNAs disrupting the LRF BS.We then compared BE strategies targeting the -200 region of the *HBG* promoters that either disrupt the LRF BS or create a *de novo* KLF1 BS in SCD HSPCs. The superior efficiency in generating the KLF1 BS by ABEs was associated with higher levels of HbF in mature RBCs and colonies derived from erythroid progenitors, compared to a strategy merely disrupting the LRF BS. Similarly, the RBC sickling phenotype was further improved in the edited samples carrying the KLF1 BS.In conclusion, we developed efficient BE strategies to disrupt repressor BS or create activator BS in the *HBG* promoters that led to therapeutically relevant HbF levels. Validation of the above-described results in HSPCs *in vivo* will provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSPCs for the therapy of β-hemoglobinopathies.

Immune Responses to AAV Vectors

85. Declining FVIII Activity Following Hepatic AAV Gene Transfer Because of Translational Shutdown Linked to an Immune Response

John S. S. Butterfield[1], Thaís B. Bertolini[2], Annie R. Piñeros[2], Kentaro Yamada[2], Sandeep R. P. Kumar[2], Jyoti Rana[2], Moanaro Biswas[2], Cox Terhorst[3], Roland W. Herzog[2]

[1]University of Florida, Gainesville, FL,[2]IU School of Medicine, Indianapolis, IN,[3]Harvard Medical School, Boston, MA

Adeno-associated virus (AAV) gene therapy aims to provide sustained replacement in diseases of protein deficiency, such as clotting factor VIII (FVIII) for the X-linked bleeding disorder hemophilia A (HA). Excitingly, liver-directed AAV-FVIII gene transfer led to complete correction of HA in clinical trials. However, these high levels of therapeutic expression were unstable and declined in years 2-4. We identified a sub-strain of BALB/c-HA mice that reliably forms an adaptive immune response against FVIII upon hepatic gene transfer with AAV8 vector expressing codon-optimized human FVIII under a liver-specific promoter. Administering mTOR inhibitor rapamycin for the eight weeks following gene transfer prevented formation of antibodies to FVIII and capsid, allowing vector readministration. Elimination of antibody formation resulted in initial FVIII activity

levels of 20-60% of normal while the mice were on immune suppression. However, in multiple experiments, average FVIII levels consistently declined to less than 5% by week 16 (with 50-100% of animals having undetectable levels, depending on the experiment). Therefore, this model allowed us to uncouple loss of FVIII expression from humoral immune responses. To counter potential cellular immune responses, we included monoclonal antibodies (mAb) in the rapamycin regimen. Combining rapamycin with a mAb to IL-15, a cytokine critical for NK and memory CD8 T cells, modestly preserved FVIII activity. We confirmed the effectiveness of blocking IL-15 signaling, and in fact improved upon this approach, by combining rapamycin with a Fc-silent mAb to the shared IL-2/IL-15 receptor beta chain (CD122). Eight weeks after immune modulation was stopped, livers of mice given anti-CD122 were still NK-depleted. In addition, Kupffer cells had lower expression of inflammatory molecules, suggesting that the improvement in FVIII activity levels by IL-15 blockade was due to suppression of an inflammatory immune response. Furthermore, we found CD8 T cell depletion with anti-CD8 after rapamycin treatment reduced the number of mice that entirely lost FVIII activity similarly to anti-IL-15 and anti-CD122 treated cohorts, indicating a contribution of CD8 T cells. However, the remaining FVIII activity was less than 5%. Together, the results suggest that multiple cell types contribute to the immune response, including CD8 T and NK cells. IHC stains revealed that rapamycin-treated animals that lost FVIII activity had no FVIII protein expression in the liver, while

those that had circulating levels due to IL-15 blockade showed FVIII expression in hepatocytes. Among animals that received immune suppression, mRNA levels were similar regardless of FVIII protein expression, and CD8 T cell depletion only modestly increased vector copy numbers compared to other rapamycin-treated animals. Interestingly, all transduced mice retained AAV copies that positively correlated with hFVIII mRNA levels but not FVIII protein expression. Mice that received vector without immune suppression—thus formed antibodies against FVIII—had 2-3 times higher gene copy numbers and mRNA levels but showed only few FVIII expressing hepatocytes. Therefore, expression was lost primarily because of a translational shutdown that could in part be prevented by dampening cellular immune responses. We are now investigating whether an unfolded protein response to FVIII contributed to the shutdown and the inflammatory response. A model of gradual loss of FVIII expression largely due to translational shutdown with a lesser contribution of hepatocellular injury/cytotoxicity would explain the observations in clinical AAV-FVIII gene therapy.

86. Requirements for Cross-Presenting Dendritic Cells and CpG Motifs in CD8$^+$ T Cell Response to AAV Gene Transfer

Thais B. Bertolini[1], Jamie L. Shirley[2], Sandeep Kumar[1], Xin Li[1], Irene Zolotukhin[3], Roland Herzog[1]

viral DNA, trigger innate immune receptors and induce AAV capsid specific CD8$^+$ T cells. TLR9 sensing of CpG motifs in the AAV vector genome has been implicated in the literature to serve as a signal for activation of CD8$^+$ T cells. For instance, TLR9 signaling in plasmacytoid dendritic cells (DCs) leads to IFN I production, which in turn licenses conventional DCs to prime anti-capsid CD8$^+$ T cells. XCR1$^+$ DCs are most efficient in cross-presenting antigen to CD8$^+$ T cells and are comprised of organ-resident CD8α$^+$ DCs and migratory CD103$^+$ DCs. We quantified the capsid-specific CD8$^+$ T cell response by tetramer stain upon intramuscular (IM) injection of AAV2 vector (1x10^{11} vg/ mouse) containing an ovalbumin-derived surrogate epitope in C57BL/6 mice. To determine the putative role of these DCs, we used XCR1-DTR mice to deplete XCR1$^+$ DC by administration of diphtheria toxin (DT). Alternatively, we treated WT C57BL/6 mice with neutralizing antiCD103. C57BL/6 mice treated with anti-CD103 showed a significantly lower frequency of capsid-specific CD8$^+$ T cell response compared with controls. Depletion of XCR1$^+$ DCs gave a more dramatic outcome and resulted in nearly complete elimination of the response. Next, we evaluated the role of CpG motifs in the immune response. For that, a vector was constructed based on an entirely CpG-free expression cassette. It contained a CpG-free edited sequence of the coding region for human coagulation factor IX (FIX) and a synthetic intron under transcriptional control of a CMV enhancer/EF1α promoter combination and SV40 polyA signal. This cassette was inserted in between AAV2 ITRs and packaged into the AAV2- SIINFEKL capsid. C57BL/6 mice received IM injections (1x10^{11} vg/mouse) of AAV2SIINFEKL depleted of CpG motifs (AAV2-SIINFEKL-CpG$^-$) or CpG rich control vector (AAV2-SIINFEKL-CpG$^+$; containing native, not CpG depleted sequences). AAV-SIINFEKL-CpG$^-$ administration markedly reduced anti-capsid CD8$^+$ T cell response as observed by the decrease of % tetramer$^+$ CD8$^+$ T cells. In contrast, we did not observe differences in capsid-specific antibody formation between groups. Next, we evaluated the immune response to FIX in male hemophilia B (C3H/ HeJ F9$^{-/Y}$) mice injected IM with AAV1-CpG$^+$ or AAV1-CpG$^-$ vector (1x10^{11} vg/mouse, n=5/group). AAV1-CpG$^-$ induced a substantially reduced (~8 fold), but not completely absent, CD8$^+$ T cell infiltration and ~2 fold higher numbers of hFIX expressing muscle fibers, as quantitated by image analysis of immunofluorescent stained sections of skeletal muscle. We conclude that XCR1$^+$ DCs are the critical subset of conventional DCs that presents AAV capsid antigen to CD8$^+$ T cells, which in part occurs through XCR1$^+$CD103$^+$ DCs. Furthermore, CpG motifs are a major activation signal for CD8$^+$ T cell responses against capsid and transgene product in AAV muscle gene transfer but are not a major factor for antibody formation.

87. Defining and Overcoming Preexisting T-Cell Adaptive Immunity to SaCas9 CRISPRCas Genome Editors

Andrea T. Lee

Hematology, St. Jude Children's Research Hospital, Memphis, TN

In vivo CRISPR-Cas genome editing has the potential to transform human medicine by directly correcting disease-causing mutations in affected cells in the body. Cas9 from *Staphylococcus aureus* (*S. aureus;* SaCas9) was the first orthologue discovered with high activity in mammalian cells that is small enough to be encoded in an AAV vector and has shown great clinical potential in preliminary *in vivo* studies.

One major concern regarding the durability of promising *in vivo* genome editing therapeutic strategies using Cas9 is the potential for immune rejection of Cas9-expressing edited cells. Pre-existing adaptive T cell immunity to the Cas9 variants derived from *Streptococcus Pyogenes* (SpCas9) and S.*aureus*, common human pathogen, have been reported. While SaCas9-specific T cells were identified, the adaptive T cell immune response to SaCas9 has not been fully characterized. Here we screened peripheral blood mononuclear cells (PBMCs) from healthy HLA-typed donors using an IFN-γbased enzyme-linked immune absorbent spot (ELISPOT) assay using a peptide library consisting of 15 amino acid long peptides with an 11 amino acid overlap that covered the entire coding region of SaCas9 to sensitively measure the SaCas9specific T cell response. We found that 24% (8 of 33) of donors screened exhibited

[1] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN,[2]Department of Medicine, University of Colorado, Aurora, CO,[3]Department of Pediatrics, University of Florida, Gainesville, FL

Gene therapy using adeno-associated virus (AAV) has emerged as one of the most promising treatments for various genetic diseases.

However, the development of immune response to the viral vector or the transgene product might compromise the outcome for long-term success. Immune stimulatory CpG motifs, which are unmethylated in

a positive T cell response to SaCas9 that was CD4-restricted in 2 of 2 analyzed positive donors. Next, we performed an unbiased epitope screen to map immunodominant epitopes using our SaCas9 peptide library for two donors. For one donor, we defined the minimal 13 amino acid epitope that was recognized by SaCas9-specific CD4 T cells. Next, we performed alanine scanning of the minimal epitope and identified 7 positions that significantly reduced T cell recognition. We engineered corresponding alanine mutations into SaCas9 to determine whether these SaCas9 variants would retain high genome editing activity. Six of 7 SaCas9 alanine variants-maintained genome editing activity at levels comparable to wild-type SaCas9. In conclusion, our results illuminate the prevalence and type of T cell-mediated immunity towards SaCas9 and offer a proof-of-principle solution of engineering immune silent variants to overcome the challenge of preexisting adaptive T cell immunity to CRISPR-Cas genome editors.

88. Clinical Outcomes in Patients with and without Pre-Existing Neutralizing Antibodies to the Vector: 6 Month Data from the Phase 3 HOPE-B Gene Therapy Trial of Etranacogene Dezaparvovec

Michael Recht[1], Frank W.G. Leebeek[2], Wolfgang Miesbach[3], Nigel S. Key[4], Susan Lattimore[1], Giancarlo Castaman[5], Eileen K. Sawyer[6], David Cooper[6], Valerie Sier-Ferreira[7], Steven W. Pipe[8]

[1]The Hemophilia Center at Oregon Health & Science University, Portland, OR,[2]Erasmus MC, University Medical Center, Rotterdam, Netherlands,[3]University Hospital Frankfurt, Frankfurt, Germany,[4]University of North Carolina, Chapel Hill, NC,[5]Center for Bleeding Disorders and Coagulation, Careggi University Hospital, Florence, Italy,[6]uniQure Inc., Lexington, MA,[7]uniQure BV, Amsterdam, Netherlands,[8]University of Michigan, Ann Arbor, MI

Introduction: Etranacogene dezaparvovec is an investigational gene therapy for hemophilia B (HB) comprising an adeno-associated virus serotype 5 (AAV5) vector containing a codon-optimized Padua variant human factor IX (FIX) gene with a liver specific promoter. Although most gene therapy clinical studies exclude participants (pts) with pre-existing neutralizing antibodies (NAb) to the capsid serotype, early clinical studies and nonhuman primate data suggest that generally prevalent titers of anti-AAV5 NAbs may not preclude successful transduction with etranacogene dezaparvovec. **Aims and methods:** A Phase 3, Health Outcomes with Padua gene; Evaluation in Hemophilia B (HOPE-B; NCT03569891) was established to further assess efficacy and safety of etranacogene dezaparvovec in adults with HB. Adult males with severe or moderate-severe HB (FIX≤2%) on prior routine FIX prophylaxis were enrolled into this open-label, single-dose, single-arm, multinational trial. Pre-existing NAbs to AAV5 were assessed but not exclusionary. Participants entered a ≥6 month lead-in period, then received a single dose of etranacogene dezaparvovec ($2x10^{13}$gc/kg) without prophylactic immunosuppression. The co-primary endpoints are change in FIX activity at 26 and 52wks and 52wk annualized bleeding rate compared to lead in. Here, outcomes at 26 weeks in participants with and without pre-existing NAbs to AAV5 are analyzed using descriptive statistics and a correlation analysis. **Results:** 54 participants were dosed and completed 26 wks of follow-up, 31 (57.4%) had no AAV5 NAbs. Of the 23 (42.6%) with AAV5 Nabs at baseline (BL), the median titer was 56.9 (1^{st}-3^{rd} quartile 23.3-282.5) with a distribution representative of the general population. The max NAb titer was 3212. One participant with a NAb titer of 198 received a partial dose and was excluded from the assessment of NAb impact on efficacy. A single participant with a NAb titer of 3212 did not respond and remained on prophylaxis. All other participants (n=52) discontinued prophylaxis and remain prophylaxis-free at 26 weeks. No correlation of pre-existing NAbs with FIX activity was observed up to a titer of 678 (n=52, r=-0.28 [95% CI -0.51, 0.00], R^2=0.078). Mean FIX activity at 26 weeks was 32.7 IU/dl (min <2, max 90.4, 1^{st}-3^{rd} quartile 16.3-42.6, n=22) in participants with NAbs versus 41.3 IU/dl (min 8.4, max 97.1, 1^{st}-3^{rd} quartile 31.3-52.7, n=31) in those without. Most common treatment-related AEs were transient transaminitis requiring corticosteroids (2/23 pts with NAbs; 7/31 pts without), infusion-related reactions (5/23 pts with NAbs; 2/31 pts without), headache (2/23 pts with NAbs; 5/31 pts without) and influenza-like illness (4/23 pts with NAbs; 3/31 pts without). No deaths and no inhibitors to FIX were reported. **Conclusions:** FIX activity was similar in participants without and with pre-existing NAbs to AAV5 up to a titer of 678; there were insufficient data to assess a relationship with higher titer NAbs. No relationship between AAV5 NAbs and safety was observed. This study demonstrates for the first time successful treatment of patients with pre-existing NABs at generally prevalent levels with an AAV5 construct, supporting broad eligibility for AAV5-based therapies.

89. Abstract Withdrawn

90. IL-1a and IL-1b Are Essential for Inflammasome Independent CD8$^+$ T Cell Responses to Hepatic AAV Gene Transfer

Sandeep Kumar[1], Moanaro Biswas[1], Annie R. Piñeros[1],

[1] Pediatrics, Indiana University, Indianapolis, IN,[2]Gastroenterology and Hepatology, Weill Cornell Medicine, New York, NY

We previously demonstrated that liver directed gene therapy with low dose adeno-associated virus serotype 8 (AAV8) vector elicited a CD8$^+$ T cell response to the transgene product, resulting in the loss of expression in murine liver. Further, we showed that these CD8$^+$ T cell response were independent of TLR9 sensing, instead relying on the IL-1R1/MyD88 pathway and CD4$^+$ T cell help. Using proliferation of adoptively transferred, CellTrace violet labeled transgene specific CD8$^+$ T cells as a readout, we demonstrated that cross presenting

Ype P. De Jong[2], Roland W. Herzog[1]

$CD11c^+XCR1^+$ DCs are critical for MHC I antigen presentation. In the present study we sought to delineate the role of IL-1α, IL-1β and the role of inflammasomes in mediating cellular responses to the AAV encoded transgene. Further, we studied kinetics of $CD4^+$ and $CD8^+$ T cell activation following AAV mediated hepatic gene delivery. Since IL-1R1 is a cognate receptor for both IL-1α and IL-1β, we used antiIL-1α and anti-IL-1β to block IL-1 signaling. Wildtype (WT) C57BL/6 mice (n=8/group) were treated with anti-IL-1α, anti-IL-1β or both antibodies one day prior to intravenous (IV) injection with AAV8OVA ($1x10^9$ vg/mouse). Treatment was continued 2x/week for four weeks. Control WT-C57BL/6 mice (n=8) received only AAV8-OVA. PBMC were screened for OVA-specific $CD8^+$ T cells using class I MHC tetramer after 4 weeks. In order to study the role of inflammasomes, knockouts mice of specific inflammasomes (NLRP1, NLRP3, AIM-2 and Caspase-1) on C57BL/6 background (n=8) were injected with AAV8-OVA and OVA-specific $CD8^+$ T cells were quantified. To understand the activation kinetics of $CD4^+$ and $CD8^+$ T cells, AAV8OVA injected C57BL/6-WT mice were euthanized on days 3, 7, 10 and 14 (n=5). $CD4^+$ and $CD8^+$ T cells from liver, hepatic lymph nodes and spleen were further analyzed for expression of activation markers (CD44, CD69 and CD107a). Naive C57BL/6-WT mice (n=5) were used to obtain baseline status of $CD4^+$ and $CD8^+$ T cells. Consistent with earlier findings, 75% of control WT mice developed a $CD8^+$ T cell response to OVA. Blocking of either IL-1α or IL-1β reduced the number of mice (33% and 50% respectively) that developed a $CD8^+$ T cell response. Moreover, only 18% of mice developed a $CD8^+$ T cell response when both IL-1α and IL-1β were blocked. These results indicate that both IL-1α and IL-1β play a critical and in part redundant role in mediating $CD8^+$ T cell response to an AAV encoded transgene during hepatic gene delivery. Since inflammasomes are an important mediator of IL-1β driven immune responses, we tested if any of the inflammasomes were required. Similar to WT mice, knockout mice for different inflammasome developed OVA specific $CD8^+$ T cell response indicating that these cellular responses were independent of inflammasome machinery. Following AAV8-OVA administration, increased expression of CD44, CD69 and CD107a was observed on $CD4^+$ and $CD8^+$ T cells isolated from liver as compared to hepatic lymph nodes or spleen. Activated $CD4^+$ and $CD8^+$ T cells were observed in the liver as early as day 3 post AAV administration. In summary our results suggest that during AAV mediated liver gene transfer, both IL-1α and IL-1β mediates $CD8^+$ T cell activation to AAV encoded transgene, which is independent of any inflammasome machinery. Further, our results imply that these cellular responses are initiated in the liver.

91. Novel miRNA-Binding Sites That Recruit miR-652 and miR-223 in AAV Vector Designs Boost Transgene Levels and Synergistically Suppress Cell-Mediated Immunity

Manish Muhuri[1,2,3], Wei Zhan[1,2,3], Yukiko Maeda[1,3,4], Jia Li[1,2], Anoushka Lotun[1], Jennifer Chen[1], Phillip W. L. Tai[a1,2,3], Guangping Gao[a1,2,5]

[1]University of Massachusetts Medical School, Horae Gene Therapy Center, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]VIDE Program, University of Massachusetts Medical School, Worcester, MA,[4]Department of Medicine, University of Massachusetts Medical School, Worcester, MA,[5]Li Weibo

Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA

Recombinant adeno-associated virus (AAV) vector gene therapy offers tremendous promise for the treatment for a variety of genetic diseases. Regulatory approvals for the treatment of two genetic diseases have already been received and clinical development for many more are on the horizon. Exciting advancements have been made in the gene delivery technologies, from the identification of novel AAV serotypes, to the development of novel vector delivery techniques. One of the challenges for long-term success of gene therapy is the development of immune response to the transgene product. This effect is attributed to the undesirable transduction of antigen presenting cells (APCs), which in turn triggers host immunity towards rAAV-expressed transgene products. miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing immunogenicity. Skeletal muscle has been considered a viable target for AAV vector-mediated gene transfer to achieve sustained production of secreted therapeutic proteins. We have previously shown that miR-142 mediated detargeting allows continued expression of transgene in myofibers, represses cytotoxic T cell response and blunts the activation of co-stimulatory signals. However, the combinatorial effect of more than one miRNA binding site in the 3'-UTR of the transgene on anti-transgene immunity has not been reported previously. In this study, we performed *in vitro* screening of binding sites for 26 miRNAs that were selected based on their high expression levels in APCs, such as dendritic cells (DCs) and macrophage cell lines, but low in myoblasts. We identified two novel miRNA binding sites, miR-652BS and miR-223BS, that are efficient at APC detargeting *in vitro*, either individually or in combination with miR-142BS. Intramuscular administration of rAAV1 vectors containing either miR-142+652BS or miR-142+223BS demonstrate higher transgene expression in skeletal myoblasts as compared to previously published detargeting constructs, with negligible antiOVA IgG production. Immunophenotyping of cells isolated from liver, spleen and muscle tissues revealed suppression of DC and costimulatory signals, and macrophage activation. In addition, there was a marked reduction in OVA specific CD8+ T cell response in those tissues accompanied by a reduction in the production of inflammatory cytokines, TNFα and IFNγ. Moreover, we present evidence that miR142-, miR-652-, and miR-223-mediated detargeting also leads to significant repression of Th17 response *in vivo*. Transgene detargeting mediated by the combination of miR-142BS and miR-652BS within the same vector cassette proves to be the most efficient at muting transgene specific immunity. Our approach, thus, advances the efficiency of miRNA-mediated detargeting to achieving synergistic reduction of transgene-specific immune responses and the development of safer and more efficient delivery vehicles for gene therapy.

Novel Factors in AAV Transduction and AAV Genomes

92. Chemical Mediated Recruitment of Epigenetic Modifiers Regulate AdenoAssociated Virus Episomal Transgene Expression

Jessica Umana[1], Liujiang Song[2], Xufen Yu[3], Jian Jin[3], Matthew L. Hirsch[2], Nathaniel Hathaway[1]

[1]Chemical Biology and Medicinal Chemistry, University of North Carolina - Chapel Hill, Chapel Hill, NC,[2]Gene Therapy Center, University of North Carolina

- Chapel Hill, Chapel Hill, NC,[3]Icahn School of Medicine, Mount Sinai, New York,

NY

Optimistic clinical data using AAV gene therapy has been observed for disorders of the muscle, blood, brain, and those affecting vision. In all these applications, the AAV vectors administered to humans have one thing in common: they are uncontrollable at the level of transgene expression. Following transduction, AAV vector genomes form circular concatemers and limited studies have demonstrated these episomes associate with histones, as well as transcriptional activators and repressors. These observations suggest a formal possibility that AAV episomes are, in part, restricted for transgene expression, alluding to the ability to modulate their epigenetic composition to enhance and/or repress the transcriptional activity. Based on these observations, it was hypothesized that AAV transgene expression can be controlled at the episomal level through targeted recruitment of epigenetic regulators of gene expression. We have developed a technology, known as chemical epigenetic modifiers (CEMs), that utilizes chemically induced proximity and endogenous epigenetic proteins to regulate specific chromosomal gene expression. The CEM technology was adapted to AAV vectors (termed AAV-CEMtrol) to investigate transgenic episome regulation post-transduction. Following AAV2 or AAV8-CEMtrol transduction in vitro, the exogenous addition of different CEMs that recruit distinct transcriptional activators (CBP/p300 or BRD4) to the recombinant AAV episome demonstrated dose-dependent and highly specific rAAV-borne reporter induction. These novel observations in well controlled and rigorous experiments demonstrate unambiguously, that AAV episomes are naturally restricted for expression in human cells and allude to the ability to enhance and potentially repress therapeutic transgene expression at a fixed AAV vector dose following in vivo administration. Towards that end, pharmacokinetic studies following a single intraperitoneal injection demonstrated the safety and bioavailability of the leading CEM in multiple tissues. Future experiments include mechanistic ChIP analyses to elucidate the exploited biology as well as performance investigations of AAVCEMtrol regulation in vivo in response to CEM administration. These data demonstrate the ability to significantly induce AAV transgene expression using particular CEMs that can recruit epigenetic regulatory

machinery to transgenic episomes. This novel approach may provide a novel approach to activate and potential repress AAV transgene expression towards controllable, and thus safer, AAV gene therapeutics.

93. The Human Silencing Hub (HUSH Complex) Is a Potent Regulator of AAV Transgene Silencing

Madhuvanthi Vijayan, Anshuman Das, Grace Stafford, Zachary Elmore, Eric Walton, Aravind Asokan

Duke University, Durham, NC

Therapeutic transgenes delivered by recombinant AAV (rAAV) vectors are known to be subject to transgene silencing as observed in several clinical trials. This poses a challenge towards achieving efficient gene expression, often requiring high vector doses. However, the latter has shown the potential to cause dose-dependent liver toxicity amongst other adverse effects. Importantly, the underlying silencing mechanisms impacting rAAV transgene expression are not well understood. In order to identify potential cellular pathways involved in rAAV transgene silencing/expression, we screened a panel of host factors known to be involved in epigenetic regulation of foreign DNA. This targeted screen revealed novel restriction factors of rAAV transduction, particularly components of the HUSH (HUman Silencing Hub) complex. The HUSH complex is known to be associated with H3K9me3-dense genomic regions and functions to repress cellular genes. The HUSH complex has also been shown to transcriptionally silence unintegrated retroviral genomes by recruiting NP220 to the viral DNA followed by methyltransferases and deacetylases. Here, we show here that CRISPR KO of individual HUSH complex members such as MPP8, PPHLN1, TASOR, SETDB1 and NP220 leads to a robust increase in AAV mediated transduction, both at the level of mRNA transcripts and translated protein. This observation was independent of AAV capsid serotype, promoter elements and observed in case of both single stranded and self-complementary rAAV vectors. Interestingly, rAAV transduction leads to increased expression of HUSH complex members like TASOR and PPHLN1, implying a role for host sensing mechanisms. Interaction of NP220 with the rAAV genome and how this changes the epigenetic landscape is currently under investigation. Lastly, we show that pharmacological inhibition of deactylases enhances rAAV transgene expression. Our approach underscores the importance of understanding host cell mechanisms that can silence rAAV transgenes. Moreover, these findings provide a roadmap towards exploring druggable aspects of rAAV genome expression and engineering the rAAV genome to potentially circumvent silencing.

94. Effects of Sexual Dimorphism and Genetic Background on AAV Tissue Transduction in Mice Following Intravenous Administration of a Diverse Capsid Pool

Elad Firnberg, Jenny M. Egley, Chunping Qiao,

Samantha A. Yost, April R. Giles, William M. Henry, Karolina J. Janczura, Justin Glenn, Devin S. McDougald,
Kirk Elliot, Randolph Qian, Subha KarumuthilMelethil, Ye Liu, Olivier Danos, Andrew C. Mercer
Gene Transfer Technologies, REGENXBIO, Inc., Rockville, MD

Introduction: The field of AAV gene therapy is quickly growing from treating small numbers of patients with rare diseases to more common clinical indications that affect larger populations. However, the literature on the effects of sexual dimorphism and genetic background on AAV transduction is limited to a small number of serotypes. In this study, we sought to characterize the tissue transduction properties of 118 pooled AAV capsids in male and female mice as well as male *mdx* mice (C57BL/10ScSn-*DMD*^{mdx}/J), a common model for Duchenne muscular dystrophy. **Results**: The AAV capsid pool consisted of 118 vectors, comprising both naturally occurring serotypes and engineered variants, with a barcoded CAG-GFP transgene. The AAV pool was administered by intravenous injection at a dose of 1.77 x 10^{13} GC/kg to female C57BL/6 (n=5), male C57BL/6 (n=5), and male *mdx* (n=5) mice, all six to eight weeks old. Vector genome copies (GC) per diploid cell and transcripts per µg RNA were quantified by digital PCR with primers for GFP and mouse glucagon as an endogenous genome control. Next generation sequencing was used to assess the relative transduction of all 118 capsids. Our results indicate that transduction differences by sex are tissue-specific and similar trends were observed across all AAV capsids and clades. In muscle tissues, we saw a 9-fold increase in GC/cell in the heart and 2-fold increase in gastrocnemius in female compared to male mice. By contrast, in liver we found a 2-fold increase in GC/cell in male compared to female mice. Male C57BL/6 mice had higher GC/cell in muscle tissues, including gastrocnemius, tibialis anterior, triceps, and diaphragm, compared to *mdx* mice. Surprisingly, this trend was highly reversed at the RNA expression level with a 24fold increase in transcripts/µg RNA in *mdx* tibialis anterior, 8-fold in *mdx* gastrocnemius, and 2-fold in *mdx* heart compared to C57BL/6 male mice. In liver, both male C57BL/6 and *mdx* mice had similar GC/cell; however, C57BL/6 male mice had 3-fold higher transcripts/ µg RNA compared to *mdx* mice. **Conclusions**: Differences in AAV gene transfer efficiency by sex or genetic background were found to be tissue dependent. Furthermore, trends in vector genome quantity and RNA expression level were not always concordant. Thus, both sex and genetic background of animal models should be considered in the development of AAV gene therapy.

95. High Throughput Screening of Diverse Mini-Promoter Libraries within AAV via Expression Linked Promoter Selection (ELiPS)

Kazuomori K. Lewis[1], Joost van Haasteren[1], David V. Schaffer[2]
[1] Bioengineering, UC Berkeley, Berkeley, CA,[2]Chemical and Biomolecular Engineering, Bioengineering, and Neuroscience, UC Berkeley, Berkeley, CA

The limited carrying capacity of the AAV genome (4.7 kb) necessitates the careful design of transgenes to maximize gene therapy efficacy and safety. A major design component of the transgene is the promoter, which dictates when, where, and how much of a therapeutic payload is expressed. Some applications require strong, ubiquitous expression, yet the common constitutive promoters - such as CMV (>750 bp) and CAG (>1600 bp) - are moderately sized and thus place limitations on the rest of the vector genome (e.g. Cas9). Truncated versions of these promoters are typically significantly weaker. Other applications call for cell or tissue specific expression, yet available specific promoters tend to be large and weaker in expression than constitutive promoters. We have developed a platform for the efficient generation of large (>10^7) libraries of synthetic promoters that can be functionally screened using AAV vectors for the high throughput selection of promoters based on their expression properties in cells or tissues of interest. We initially focused on engineering small (~200 bp), strong ubiquitous promoters for use in AAV-mediated gene delivery. To date, the largest screen of synthetic promoters in mammalian cells has been a microprinted array of 52,000 library members. We have developed a method termed ELiPS (Expression Linked Promoter Selection) in which synthetic promoters are built sequentially from small motifs in coordinated steps, allowing precise control of promoter size. ELiPS enables the construction of synthetic promoter libraries in which a barcode in the 3` UTR of the mRNA transcript is directly linked to the identity of the promoter that drove its expression, a design that is amenable to next generation sequencing analysis of promoter strength. Based on bioinformatic analysis of gene expression databases, we engineered two large libraries containing over 5x10^7 short (~200 bp) synthetic promoters intended for ubiquitous, AAV-mediated gene expression. Through just a single round of selection in an initial experiment in HEK 293T cells, we have identified a synthetic promoter of 218 bp termed ELiPS-L2-293T.1 (EL2T.1) that has 76% of the activity of the CAG promoter (1664 bp) and 82% of the activity of the CMV promoter (808 bp) - via flow cytometry, MFI_{CAG} 8570 ± 611, MFI_{CMV} 7985 ± 1128, $MFI_{EL2T.1}$ 6583 ± 1118. The expression level of EL2T is also not statistically significantly different from that of CMV ($p = 0.159$, twotailed Student's t-test, unequal variance). This approach is currently being applied to screen for small, strong, constitutive promoters in human tissue models. Our ELiPS platform is thus highly effective in the generation and high throughput screening of large synthetic promoter libraries. We are now in the process of applying our platform technology to identify strong, short promoters in human *in vitro* culture models and furthermore anticipate its translation *in vivo* to derive tissue and cell-specific promoters.

96. GMEB2 is a Conserved Cellular AAV Restriction Factor That Inhibits Transduction of Human Stem Cells

Amanda M. Dudek, Nicole M. Johnston, Sriram Vaidyanathan, Sridhar Selvaraj, Matthew H. Porteus
Pediatrics, Stanford University, Palo Alto, CA

Cellular factors that inhibit AAV transduction are of great interest to identify areas of improvement for AAV-based gene therapies for both episomal AAV expression as well as AAV-mediated genome editing. Here, we have identified the P79 component of the parvovirus initiation factor (PIF) complex, GMEB2, as a highly conserved AAV restriction factor. Knock-out of GMEB2 increases transduction in cell lines and primary cells across all tested AAV capsids including AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV9, and AAVDJ and at both high and low genome copy number, demonstrated by an up to 10-fold increase in GFP positive cells. At high transduction levels (above 90% GFP+ in WT cells), MFI is drastically increased demonstrating a potential of GMEB2 modulation to increase not only overall number of transduced cells, but also expression level within the cell. Increased transduction in GMEB2 KO cells is observed for constructs with a variety of promoters, such as CMV and UBC demonstrating that this restriction is specific to the AAV sequence. GMEB2 is highly expressed in primary cell types that are inefficiently transduced by AAV including human induced pluripotent stem cells and human airway stem cells. In contrast, CD4+ T cells and hematopoietic stem and progenitor cells (HSPCs) which are more efficiently transduced by AAV express little or no detectible GMEB2. Using a highly efficient multiplexed guide RNA/Cas9 strategy for knock-out, we demonstrate that loss of GMEB2 increases transduction in clinically relevant primary human airway bronchial stem cells which we have previously used for AAV-mediated genome editing as a corrective therapy for Cystic Fibrosis. Subcellular fractionation and western blot experiments demonstrate that although GMEB2 is reported to be a DNA-binding protein, restrictive cell types express high amounts of cytoplasmic and/or membrane associated GMEB2 rather than nuclear-localized GMEB2. Droplet digital PCR from knock-out cell fractions transduced with AAV demonstrate an increase in overall genome number in the lysates, while maintaining the overall genome distribution in cytoplasmic vs. membrane vs. nuclear fractions, suggesting that the restrictive effect of GMEB2 occurs at an early stage in the entry pathway. Additionally, in the presence of nucleofection the beneficial effect of GMEB2 KO is lost, suggesting that nucleofection at the time of AAV transduction may bypass the restrictive activity of GMEB2. These studies demonstrate a highly conserved AAV restriction factor which is capsid independent and influences the transduction efficiency of primary human stem cells.

97. Rationally Designed Inverted Terminal Repeats Improve AAV Vector Production

Liujiang Song[1,2], Zhenwei Song[1], R. Jude Samulski[1,3], Matthew L. Hirsch[1,2]

[1]Gene Therapy Center, University of North Carolina, Chapel Hill, NC,[2]Department of Ophthalmology, University of North Carolina, Chapel Hill, NC,[3]Department of Pharmacology, University of North Carolina, Chapel Hill, NC

Gene delivery approaches using Adeno-associated virus vector (AAV) are currently the leading method of human gene therapy. In all clinical AAV gene therapy applications, the only conserved viral DNA sequences in recombinant AAV (rAAV) are based on the inverted terminal repeats of serotype 2 (ITR2), which are required for multiple aspects of the AAV life cycle such as genome replication and encapsidation. Our laboratory has engineered a panel of functional synthetic ITRs (synITRs) via rational nucleotide substitutions and deletions of putative host factor binding sites. To assess the influence of ITR sequence variations, transgenic genomes flanked by ITR2 or synITRs were evaluated for rAAV production. Additionally, several biological characteristics were analyzed including: 1) the impact of the ITR sequences on rAAV genome replication, 2) the packaging efficiency and genomic integrity, and 3) overall rAAV titers. This study

also considered the impact of the synITR sequences on inherent ITR transcriptional activity and exogenous promoter function following rAAV transduction. Derived data demonstrated a phenomenon of ITR sequence-dependency on rAAV yield, as well as the capsid:genome packaging ratio. Notably, vectors exhibiting synITR257, an ITR2 size-matched sequence in which selected guanine-cytosine rich regions were substituted with adenine-thymine nucleotides, showed a significant three-fold increase in vector production compared to ITR2-based rAAV as determined by probe-based qPCR, supported by alkaline gel electrophoresis, and capsid packaged ITR sequencing. The hypothesis of increased transgenic genome replication to account for the increased rAAV yield was refuted as results indicate that vector genomes employing synITR257 replicate less efficiently than ITR2flanked genomes in production cells. This is the first evidence showing that transgenic genome replication is not a rate-limiting step for rAAV production using 293 triple transfection production methodology. Interestingly, synITR257 significantly enhanced the encapsidation efficiency of rAAV vectors determined by the capsid:genome ratio compared to ITR2. Furthermore, preliminary results suggest that the synITRs and ITR2 may have different inherent transcriptional activity following transduction with a promoter-less luciferase transgene cassette. However, in the context of a strong exogenous promoter, reporter activity was not significantly different between synITR257 and ITR2 following transduction of the evaluated cell lines. The collective data demonstrate that rAAV transgenic genome replication is not rate-limiting for rAAV production and that modulation of ITR sequences influence rAAV yield, potency, and thus represents another optimization step for enhanced and potentially safer AAV-based gene therapy drugs.

98. Characterization of AAV Inverted Terminal Repeats by Atomic Force Microscopy

Marianne Laugel*[1], Yeraldinne Carrasco-Salas*[2], Blandine Simon[2], Mathieu Mevel[1], Oumeya Adjali[1], Cendrine Moskalenko#[2], Magalie Penaud-Budloo#[1]

[1]INSERM UMR 1089, University of Nantes, Nantes, France,[2]UMR 5672, ENS de Lyon, Lyon, France

The rational design of a recombinant adeno-associated vector (rAAV) consists of removing the three open reading frames of the wild-type AAV and cloning a sequence of interest between the two inverted terminal repeats (ITR). Hence, ITRs are the only viral elements kept in the AAV vector genome. AAV ITRs are 143-167 nucleotides in length. These viral telomeres are composed of

imperfect palindromic regions folding into a particular T-shaped structure. Essential for vector production, they contain the minimal elements required *in cis*for rAAV genome replication and encapsidation. With the numerous successes of gene therapy clinical trials and the commercialization of AAV-based drugs, there is a need for better understanding and characterizing these vectors. In particular, ITRs have been shown to be involved in cell-specific transcription, leading to renew interest in these fascinating sequences. In this work, we have developed a protocol allowing the visualization of AAV vector genomes by Atomic Force Microscopy (AFM), and specifically the assessment of ITR secondary structures. Single-stranded genomes flanked by wild-type AAV2 ITR were extracted from AAV vectors, incubated with Mg2+ and absorbed on a Mica surface for AFM imaging in air and in Peak Force mode. Length

and height of observed molecules were calculated using Matlab and Gwyddion programs. A majority of intact full-length rAAV genomes of approximatively 1.5 µm (4.5kb) were observed, validating our DNA extraction method. The presence of folded ITRs at the extremities of the vector genome was confirmed by height measurement. Moreover, three types of ITR secondary structures were found: a T-shape structure consistent with the most energetically stable structure predicted by RNAfold, a linear form consistent with inter-molecular annealing of unfolded ITRs and an intermediate folding conformation. Our study confirms that ITRs are dynamic structures that can adopt diverse folding structures. Controlling experimental parameters, our method could give new insights into the secondary structures of rAAV DNA, and particularly of ITRs, in different cellular compartments. Further investigations will be necessary to determine how these secondary structures could impact the rAAV molecular fate and DNA-triggered cellular responses.

Preclinical Gene Therapy for Neurologic Disorders II

99. A Novel Exon Specific U1 snRNA Therapeutic Strategy to Prevent Retinal Degeneration in Familial Dysautonomia

Anil Chekuri[1,2], Elisabetta Morini[3], Emily Logan[1], Aram Krauson[1], Monica Salani[1], Giulia Romano[4], Federico Riccardi[4], Franco Pagani[4], Luk H. Vandenberghe[4], Susan A. Slaugenhaupt[3]

[1]Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[2]Department of Neurology, Harvard Medical School, Boston, MA,[3]Department of Neurology, Harvard Medical School, Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[4]Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disorder caused by a splice mutation in the gene encoding Elongator complex protein 1 (*ELP1*, also known as *IKBKAP*). A T-to-C base change in the 5' splice site of *ELP1* exon 20 results in exon 20 skipping with tissue specific reduction of ELP1 protein predominantly in central and peripheral nervous system. In addition to complex neurological phenotype, FD patients also exhibit progressive retinal degeneration severely affecting their quality of life. To test novel splicing-targeted therapeutic approaches, we developed a phenotypic mouse model of FD, *TgFD9; Ikbkap*$^{20/flox}$ which exhibits most of clinical features of the disease while displaying the same tissue specific mis-splicing observed in patients. Here, we report a thorough characterization of the retinas of our FD mouse using SD-OCT and immunohistochemical assays during disease progression. Our findings showed a significant decrease in the thickness of the retinal nerve fiber layer (RNFL) and the ganglion cell layer (GCL) starting from 3 months of age. Retinal whole-mount analysis showed reduction of RGC cell counts from 6 months of age. Histopathological analysis of the optic nerve from FD mice using neurofilament (NF) staining indicated diffuse degeneration of axonic bundles demonstrating that our mouse model correctly recapitulates the retinal degeneration observed in patients. To restore correct *ELP1* splicing defect and rescue retinal degeneration, we have designed a novel splice targeted therapy using modified version of the spliceosomal U1 snRNAs (ExSpeU1s) that permit targeted binding to intronic sequences downstream of the mutant 5' splice site enabling efficient recruitment of spliceosomal machinery. We have analyzed the efficiency of splicing correction of the ExSpeU1s through *in vivo* delivery using adeno associated vectors (AAV). Our findings suggest that our novel FD mouse model exhibit most of retinal degeneration pathology observed in FD patients. Our *in vivo* preliminary data demonstrate the valuable therapeutic potential of ExSpeU1 RNA delivery to treat retinal degeneration in FD.

100. Novel RNA-Targeting Gene Therapy Approach for Usher's Syndrome Type II Retinitis Pigmentosa

Daniel Gibbs, Rea Lardelli, Greg Nacthrab, Daniela M. Roth, Shawn Lee, Claire Geddes, Alistair Wilson, Nandini Narayan, Dimitrios Zisoulis, Ranjan Batra

Locanabio, Inc., San Diego, CA

Mutations in the USH2A gene are the most common cause of Usher Syndrome type II (USH2) and non-syndromic autosomal recessive retinitis pigmentosa (10-15% of ARRP). Mutations in exon 13 of USH2A gene are the most common mutations in USH2A gene (~16,000 patients in the North America and EU) and lead to loss of function of the very large (600KD) Usherin (USH2A) protein. USH2A isoforms lacking exon 13 lead to the production of a slightly truncated functional protein. Recently, genome-editing and antisense oligonucleotide (ASO)-based approaches have been described to target USH2A exon 13 at the DNA and RNA levels, respectively. Antisense oligonucleotides require recurrent delivery in the intravitreal space and DNA targeting entails the risk of permanent DNA-level off-target effects. Here we describe an RNA-targeting CRISPR/Cas13d gene therapy approach for skipping USH2A exon

13. Our *in vitro* data demonstrates that our candidate shows >98% efficacy in skipping exon 13 (alternative splicing) leading to production of the slightly shortened but functional Usherin protein. Furthermore, we packaged this candidate in a single AAV gene therapy delivery vector for one-time treatment. We screened various AAV serotypes including but not limited to AAV2, AAV5, AAV7, AAV8 and AAVrh10 to optimize delivery to the photoreceptors in wild-type mice. We also constructed another version of our potential therapeutic to skip mouse exon 12 (equivalent to the human exon 13) that requires two guide RNAs along with dCas13d, which shows dose-dependent and highly efficient exon 12 skipping *in vitro*. Finally, we show photoreceptor transduction, safety (4 and 8 weeks), and molecular efficacy (exon 12 skipping) of our AAV-packaged USH2A therapeutic candidate post sub-retinal delivery *in vivo* in mice. These findings support the development of RNA-targeting gene therapies using highly efficacious CRISPR/Cas13d exon skipping in USH2A for patients with exon 13 mutations.

101. Evolution of Modified AAV Vectors in Rhesus Macaque Cochlea

Paul T. Ranum[1], Stephen R. Chorney[2], Yong Hong Chen[1], Megan S. Keiser[1], Xueyuan Liu[1], Congsheng Chen[1], Geary Smith[1], Amy Muehlmatt[1], Luis Tecedor[1], Robert C. O'Reilly[2], Beverly L. Davidson[1,3]

[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Division of Pediatric Otolaryngology, The Children's Hospital of Philadelphia, Philadelphia, PA,[3]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Introduction: Gene therapy is a powerful tool to combat hearing loss and deafness, but its clinical implementation is impeded by the temporal bone anatomy, a high diversity of genetic lesions, and myriad cochlear sensory- and supporting-cell types. The identification of adeno-associated virus (AAV) capsids capable of efficient hair-cell transduction, including Anc80L65 and AAV2, has brought cochlear gene therapy closer to a clinical reality for some forms of genetic deafness. However, for genetic lesions impacting hearing at the level of cochlear supporting cells, additional vectors with varying tropism characteristics are needed. To meet this need we implemented a directed AAV evolution screen in the nonhuman primate (NHP) cochlea to identify a library of AAV capsid variants with diverse transduction properties capable of satisfying the requirements of current and future cochlear gene therapy designs. **Methods:** Peptide modified AAV libraries were first generated by insertion of random peptides into AAV1, AAV2, and AAV9. In vivo selection was performed in two successive enrichment steps in which our AAV capsid library was delivered to tissues of interest. Tissues were then recovered and capsid variants were extracted and used as the basis for the subsequent round of in vivo enrichment. At each round of enrichment NGS amplicon sequencing libraries were generated and sequencing data was used to track enrichment of each capsid configuration. Initially, our AAV capsid library was delivered to the NHP brain by Intracerebroventricular (ICV) injection. In this experiment we observed striking and unexpected transduction of NHP cochlear hair-cells and supporting cells by AAV9 derived capsid variant. This unexpected transduction of cochlear cells following ICV injection may occur via the fluid connection of CSF to the perilymph bathing the cochlear aqueduct. This finding prompted us to test direct injection of the AAV9 variant by round window membrane injection with lateral canal fenestration (RWM+LCF) and to undertake a separate cochlea-specific AAV enrichment study in which we delivered our AAV capsid library directly to the cochlea via the same RWM+LCF method. One cochlea received direct injection of 1999 and four cochlea (two NHPs) received injections of the AAV capsid library over the course of two rounds of in vivo enrichment. **Results:** Here we report the successful delivery and selection of modified AAV vectors in the cochlea of Rhesus macaque. Using a fluorescent reporter, we observed that the AAV9 variant transduces cochlear inner hair cells effectively, though incompletely, as well as an assortment of supporting cells including cells of the stria-vascularis and spiral ganglion neurons. Sequencing results from the directed cochlear AAV evolution strategy reveal an assortment of enriched capsid variants for which small-library- and fluorescence-based validation is ongoing. Given the remarkable similarity with the human cochlea, the

Rhesus macaque animal model provides a tremendous opportunity to refine both delivery methods and identify AAV capsid variants with high potential for clinical applicability.

102. A Novel Retinal Gene Therapy Strategy for Batten Disease and Beyond

Maura Schwartz[1], Archana Jalligampala[2], Alex Campbell[3], Isabella Palazzo[3], Shibi Likhite[1], Andrew Fischer[3], Maureen McCall[2], Kathrin Meyer[1]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Kentucky Lions Eye Institute, Louisville, KY,[3]The Ohio State University, Columbus, OH

Batten Disease is a fatal, lysosomal storage disorder characterized by cognitive and motor deficits, vision impairments, and seizures. Loss of vision is a characteristic in 10 of the 13 Batten Disease subtypes. Our group has pioneered AAV9 gene therapy treatments that achieve widespread transduction of the brain and spinal cord. However, based on non-human primate data, AAV9 transduction of the retina after CSF delivery might be suboptimal. Thus, strategies to further improve retinal targeting using the same AAV9 gene therapy vector already in clinical trial could further improve Batten Disease gene therapy. Similar to most genetic ocular diseases, photoreceptor degeneration is the most commonly cited pathology in patients. However, recent studies suggest that in some subtypes of Batten Disease such as CLN3 and CLN6, expression must be rescued within cells of the inner nuclear layer (INL) of the retina to prevent vision loss. However, a full visual rescue was not achieved in mouse studies with INL targeting alone. We performed single-cell RNA sequencing in non-human primates to confirm CLN gene expression and found a generally ubiquitous, low expression in almost all cell-types of the retina. This suggests the need for a wide-ranging retina-tropic AAV that efficiently targets the INL, among other cell-types, which currently does not exist. We have recently discovered that administration of neuraminidase (NA), a sialidase enzyme, prior to or in combination with AAV9. GFP, drastically increases

transduction through all the retinal layers in the murine and porcine retina. In mice, we delivered 11 mU of Neuraminidase prior to or in combination with $2e^{10}$vg AAV9.GFP. This manipulation resulted in an increase of GFP+ cells in the murine inner retina. Hand counts of GFP+ cells co-localized with cell-specific counterstains confirmed a 40% increase in AAV9 transduction in Müller glia, a 15% increase in amacrine cells, a 30% increase in bipolar cells, and a 78% increase in horizontal cells. Moreover, we translated this promising treatment paradigm to a WT porcine model. Intravitreal injections delivered 844mU of Neuraminidase prior to or in combination with $2e^{12}$ vg AAV9.GFP. Confocal imaging of retinal sections shows GFP expression in almost all, if not all, retinal cell types. This suggests that it may be possible to target every cell-type of the retina with a single AAV vector. This is especially important with respect to the treatment of Batten Disease and has many additional implications. Also, this strategy would be highly useful in the field of optogenetics, where there is a need for an efficient method to express light-sensitive opsins in cells of the INL to restore sight in individuals that have already lost their photoreceptors.

Preclinical Gene Therapy for Neurologic Disorders II

103. Reprogramming to Recover Youthful Epigenetic Information and Restore Vision

Yuancheng Lu[1], Benedikt Brommer[1], Xiao Tian[1], Anitha Krishnan[1], Margarita Meer[1], Chen Wang[1], Noah Davidsohn[1], George Church[1], Konrad Hochedlinger[1], Vadim Gladyshev[1], Steve Horvath[2], Morgan Levine[3], Meredith Gregory-Ksander[1], Bruce Ksander[1], Zhigang He[1], David Sinclair[1]

[1]Harvard Medical School, Boston, MA,[2]Department of Human Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA,[3]Department of Pathology, Yale School of Medicine, New Haven, CT

Aging is a degenerative process that leads to tissue dysfunction and death. A proposed cause of aging is the accumulation of epigenetic noise that disrupts gene expression patterns, leading to decreases in tissue function and regenerative capacity. Changes to DNA methylation patterns over time form the basis of aging clocks, but whether older individuals retain the information needed to restore these patterns— and, if so, whether this could improve tissue function—is not known. Over time, the central nervous system (CNS) loses function and regenerative capacity. Using the eye as a model CNS tissue, here we show that ectopic expression of Oct4 (also known as Pou5f1), Sox2 and Klf4 genes (OSK) through AAV2 in mouse retinal ganglion cells restores youthful DNA methylation patterns and transcriptomes, promotes axon regeneration after injury, and reverses vision loss in a mouse model of glaucoma and in aged mice. The beneficial effects of OSK-induced reprogramming in axon regeneration and vision require the DNA demethylases TET1 and TET2. These data indicate that mammalian tissues retain a record of youthful epigenetic information— encoded in part by DNA methylation—that can be accessed to improve tissue function and promote regeneration in vivo.

104. Efficacious, Safe, and Stable Inhibition of Corneal Neovascularization with rAAV-*KH902* in a Mouse Model of Corneal Alkali injury

Wenqi Su#[1,2], Shuo Sun#[1,3], Bo Tian#[1], Phillip W. L. Tai[4], Jihye Ko[4], Yongwen Luo[4], Xiao Ke[5], Qiang Zheng[5], Hua Yan*[2], Guangping Gao*[4], Haijiang Lin*[1]

[1]Department of Ophthalmology & Visual Sciences, University of Massachusetts Medical School, Worcester, MA,[2]Ophthalmology, Tianjin Medical University General Hospital, Tianjin, China,[3]Tianjin Key Laboratory of Retinal Fuctions and Diseases, Tianjin International Joint Research and Development Centre of Ophthalmology and Vision Science, Eye Institute and School of Optometry, Tianjin, China,[4]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[5]Chengdu Kanghong Pharmaceuticals Group, Chengdu, China

Introduction: Corneal avascularity is an essential prerequisite of corneal transparency. Pathological conditions such as inflammation, hypoxia, and limbal barrier dysfunction disrupt the balance of pro- and anti-angiogenic factors. Under these conditions, corneal neovascularization (CoNV) can occur, causing a decrease in corneal transparency and visual impairment. CoNV develops in over 1.4 million patients in the United States per year. Among the well-studied anti-VEGF drugs in clinical testing, periodic injections of KH902 (trade name, conbercept) can inhibit ocular neovascularization. However, more efficacious and long-lasting treatment for patients with clinical CoNV is an urgent need. To this end, we investigated the long-acting anti-angiogenesis effects and safety of rAAV-delivered *KH902* in a mouse corneal injury model. **Methods:** In this study, we packaged the *KH902* transgene into rAAV vectors, and delivered them by single intrastromal or subconjunctival injections. The transduction efficiency of vectored *KH902* and its cell tropism in the cornea were determined by immunostaining. Droplet Digital PCR was used to quantitate *KH902* mRNA expression. Potential side effects were evaluated by measuring the central corneal thickness (CCT) at various time points by using optical coherence tomography (OCT). Immunostaining of corneal sections with CD11b and F4/80 was performed to assess immune responses two weeks post rAAV-*KH902* injection. Progression of CoNV in an alkali burn-induced model was tracked and quantified at 5 and 10 days, and 2, 3, 4, 8, and 12 weeks post-administration of rAAV-*KH902* by live animal imaging microscopy. Activation of DLL4/Notch signaling and ERK, downstream of effectors of VEGF signaling, were assessed by immunostaining and Western blot. **Results:** We demonstrate that rAAVs can successfully mediate KH902 expression in corneal keratocytes after intrastromal administration, while subconjunctival administration resulted in poorer transduction of keratocytes. *KH902* mRNA expression in the

[1] Neuroscience, Voyager Therapeutics, Cambridge, MA,[2]Vector Engineering, Voyager Therapeutics, Cambridge, MA,[3]Vector Production, Voyager Therapeutics, Cambridge, MA

cornea reached peak levels at 1-2 weeks and then gradually declined. Nevertheless, *KH902* was still detectable at three months post intrastromal delivery. In addressing the safety of rAAV-*KH902* in the cornea, we found that high doses (1.6×10^{10} vg/eye) of rAAV-*KH902* triggered an intense immune response, while lower doses (8×10^{8} vg/eye) caused a minimal response. Based on this finding, we adopted the lower dose for our therapeutic studies in the corneal alkali-burn model for CoNV. We observed that rAAV-*KH902* conferred a dramatic suppression of CoNV for at least three months post-alkali burn without adverse events. In contrast, we observed that the inhibition of CoNV conferred by a single administration of the manufactured conbercept drug lasted only for 14 days post injection. Furthermore, the therapeutic outcomes produced by rAAV-*KH902* treatment were concomitant with the downregulation of DLL4/Notch signaling and ERK activation in the treated tissues. **Conclusion:** Our study demonstrates the potential and relative safety of rAAV-based, longterm anti-angiogenesis therapy with *KH902* in the treatment of CoNV. # The authors contributed equally to this work. * Corresponding authors.

105. Efficacy of a Vectorized Anti-Tau Antibody Using Systemic Dosing of a Blood Brain Barrier Penetrant AAV Capsid in Mouse Models of Tauopathies

Wencheng Liu[1], Jerrah Holth[1], Maneesha Paranjpe[1], Xiao-Qin Ren[2], Yanqun Shu[2], Giridhar Murlidharan[1], Charlotte Chung[1], Alex Powers[1], Emalee Peterson[1], Abigail Ecker[1], Usman Hameedi[3], Kyle Grant[3], Vinodh Kurella[2], Dillon Kavanagh[2], Omar Khwaja[1], Jay Hou[2], Steve Paul[1], Kelly Bales[1], Todd Carter[1]

Anti-tau immunotherapy is a promising therapy for tauopathies, including Alzheimer's disease (AD), frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP). While passive immunization with anti-tau monoclonal antibodies has been shown by several laboratories to reduce age-dependent tau pathology and neurodegeneration in mouse tauopathy models, these studies have typically used repeated high weekly doses of antibody and reported only moderate reduction of tau pathology. We have previously demonstrated broad distribution and expression of a vectorized anti-tau antibody in the mouse brain using a blood-brain barrier (BBB) penetrant AAV capsid administered intravenously (IV). This gene therapy-based approach has potential advantages, including continuous expression of antibody in the central nervous system (CNS) after a single administration of vector, increased CNS levels of tau antibody relative to passive immunotherapy, and the potential to target intracellular tau aggregates. Here we describe studies characterizing the onset of transduction following dosing with a vectorized tau antibody and its efficacy in mouse models of tauopathy. AAV vectors comprising a novel capsid and a transgene encoding an anti-tau monoclonal antibody were administered by IV bolus to wild type mice. Biodistribution and cellular tropism in the CNS were evaluated by ELISA and (or) immunostaining, and vector genome levels were quantified using digital PCR, resulting in the detection of antibody expression soon after dosing. We then investigated efficacy in reduction of tau pathology in mouse tauopathy models. Treatment with our vectorized antibody resulted in durable antibody expression in the CNS and a corresponding reduction in CNS insoluble pathological tau and neurofibrillary tangles. Our results indicate that systemic dosing of a vectorized anti-tau antibody using a BBB-penetrant AAV capsid results in reduced tau pathology and may represent a new single-dose therapeutic strategy for treating various tauopathie

Synthetic/Molecular Conjugates and Physical Methods for Delivery

106. Combinatorial Modified mRNA Induces Cardiovascular Regeneration Post Muscle Ischemic Injury

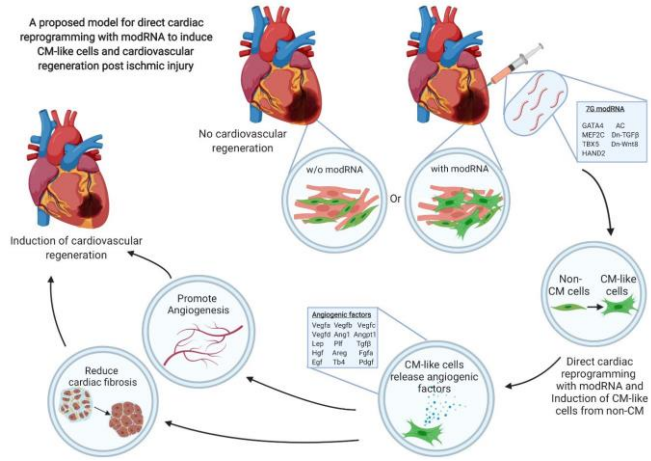
Keerat Kaur[1], Asharee Mahmood[1], Hanna Girard[1], Ann Anu Kurian[1], Magdalena Zak[1], Maria Paola Santini[1], Elena Chepurko[1], Vadim Chepurko[1], Djamel Lebeche[1], Jason C. Kovacic[1], Roger J. Hajjar[2], Shahin Rafii[3], Lior Zangi[1]

[1]Cardiology, Icahn School of Medicine, Mount Sinai, New York, NY,[2]Phospholamban Foundation, Amsterdam, Netherlands,[3]Cardiology, Weill Cornell Medicine, New York, NY

Despite advances in the preventative medicine, ischemic heart disease remains a major cause of morbidity and mortality in the industrialized world, causing significant societal and economic burden. Post myocardial infarction (MI), the adult mammalian heart has limited regenerative capacity to recover from the loss of cardiomyocytes (CM). Thus, reprogramming non-cardiomyocytes (non-CMs) into

cardiomyocyte (CM)-like cells in vivo is a promising strategy for cardiac regeneration. However, the current viral-based gene transfer delivery methods have low and erratic transduction efficiency that precludes these technologies from being translated to the clinic. Modified mRNA (modRNA)-based gene delivery features transient but potent protein translation and low immunogenicity, with minimal risk of insertional mutagenesis. Here we used a modRNA gene delivery platform to deliver different stoichiometry of cardiac-reprogramming genes (Gata4, Mef2c, Tbx5 and Hand2) together with reprogramming helper genes (Dominant Negative (DN)-TGFβ, DN- Wnt8a and Acid ceramidase (AC)), named 7G, to induce direct cardiac reprogramming post MI. In this study, we found that a combination of 7G modRNA cocktail can play a vital role in cardiac reprogramming. Compared to previously established Gata4, Mef2C and Tbx5 (GMT) reprogramming cocktail, 7G modRNA reprogrammed twice the number of nonCMs to CMs (from 28% to 57%) in vitro. Importantly, repeated 7G modRNA transfection results in beating CMs and complete cardiac reprograming *in vitro*. Using our lineage tracing model, we showed that one-time delivery of the 7G-modRNA cocktail at the time of MI partially

reprogrammed ~25% of the non-CMs in the scar area to CM- like cells. Furthermore, 7G modRNA treated mice showed significantly improved cardiac function, longer survival, reduced scar size and greater capillary density than control mice 28 days post-MI. We attributed the improvement in heart function post modRNA delivery of 7G or 7G with increased Hand2 ratio (7G-GMT Hx2) to significant upregulation of 15 key angiogenic factors without any signs of angioma or edema. Intriguingly, 7G modRNA cocktail was also able to induce to neovascularization in mouse hindlimb ischemia model, indicating that 7G-modRNA cocktail administration promotes vascular regeneration in cardiac and skeletal muscle post ischemic injury. Thus, combinatorial, first of its kind 7G modRNA cocktails presents a safe and high efficiency gene delivery approach with therapeutic potential to treat ischemic diseases.



Synthetic/Molecular Conjugates and Physical Methods for Delivery

107. Sustained Episomal Transgene Expression *In Vivo* Driven by Non-Viral DNA Delivery to Rodent Liver

Stoil Dimitrov[1], Joe Sarkis[2], Kevin Smith[3], Ben Geilich[2],
Sean Severt[2], Kevin Davis[4], Edward Acosta[4], Karen Olson[4], Molly Bell[4], Kristine Burke[4], Shan Naidu[4], Eric Jacquinet[4], Carla Leite[2], Andrew Auerbach[2], Nicholas Amato[2], Erik Owen[2], Jordan Santana[2], Tatiana Ketova[2], Brian Fritz[2], Christopher Tunkey[2], Bhargav Tilak[2], Jeffrey Pimentel[1], Mychael Nguyen[1], Susanna Canali[1], Jared Iacovelli[1], Eric Huang[1]

[1]New Venture Labs, Moderna, Cambridge, MA,[2]Platform Research, Moderna, Cambridge, MA,[3]Process Development, Moderna, Cambridge, MA,[4]Non-Clinical Sciences, Moderna, Cambridge, MA

Efficient non-viral delivery of exogenous DNA vectors *in vivo* has the advantage of decreased immunogenicity in comparison to current virally-mediated delivery approaches and largely increased payload capacity. The increased maximal size of the delivered exogenous DNA allows for the introduction of complex transcription units containing the gene of interest together with endogenous or tissuespecific promoter and enhancers, as well as additional mammalian transcription or stability control elements. Here we describe a successful non-viral, Lipid nanoparticles (LNP)-mediated delivery of non-integrating plasmid DNA vectors *in vivo* that drive hepatocytesspecific expression in rodents and allow for long-term stable episomal expression of a transgene. Plasmid DNA-mediated delivery of Firefly Luciferase gene, under control of the liver-specific Alpha-1 Antitrypsin (AAT) promoter, resulted in stable, steady-state Luc expression in mouse hepatocytes for six weeks until study termination. LNPmediated delivery of plasmid DNA encoding a secreted Fc fragment of human IgG1, driven by the liver-specific AAT promoter, resulted in sustained, dose-dependent expression of the secreted hIgG-Fc protein that lasted for six months in Sprague-Dawley rats. Importantly, plasmid DNA-mediated liver-specific transgene expression resulted in mild, transient elevation of ALT and AST six hours after dosing, which resolved by 48h post-delivery. No other changes in the comprehensive blood biochemistry profile of the test subjects were detected. We have observed transient, dose-dependent activation of Type I interferon response after plasmid DNA delivery *in vivo*. In both mouse and rat serum, levels of IFN-alpha, IP-10, MCP-1, MCP-3 and RANTES were transiently elevated at 6h post-delivery and decreased 24h to 48h post dosing. In conclusion, we have demonstrated that LNP-mediated delivery of DNA vectors *in vivo* can lead to long-term, stable tissuespecific expression of both intracellular or secreted proteins in mouse and rat hepatocytes. Expression of transgenes can last for at least 24 weeks. LNP-mediated DNA delivery resulted in mild and transient elevation of liver enzymes and serum inflammatory cytokines.

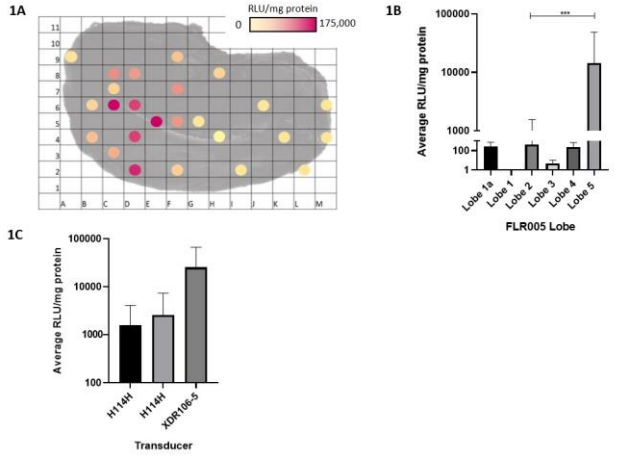
108. Optimization of Transcutaneous Ultrasound Mediated Gene Delivery into Large Animals

Megan A. Manson[1], Feng Zhang[1], Carley M. Campbell[1], Peter Kim[1], Kyle P. Morrison[2], Maura Parker[3], Keith
R. Loeb[3], Dominic Tran[1], Masaki Kajimoto[4], Rainier Storb[3], Carol Miao[1]

[1]Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Sonic Concepts, Inc., Bothell, WA,[3]Fred Hutchinson Cancer Research Center, Seattle, WA,[4]Seattle Children's Research Institute, Seattle, WA

Hemophilia A is an X-linked clotting disorder characterized by a deficiency of functional factor VIII (FVIII) protein. In previous experiments, we performed transcutaneous ultrasound mediated gene delivery (UMGD) of a FVIII plasmid into normal canines with success using the H114H focused transducer. In order to increase the treatment area, we have recently developed a new transducer, XDR106-5. This transducer is a 5-element, unfocused transducer that leverages both PNP output to circumvent tissue attenuation, as well as a larger treatment area for effective gene delivery. The unique 5-element configuration allows the user to easily scan the transducer to cover a greater treatment area as opposed to the H114 focused transducer, which has a small area of focus. In preparation for future experiments in hemophilia A canines, we aimed to optimize the surgical protocol and ultrasound parameters and tested an improved transducer design. A pGL-4 plasmid encoding the luciferase reporter gene was injected in combination with RN18 microbubbles (MBs) into the canine liver lobes through a fluoroscopy-guided balloon

catheter (either a 12 mm x 20 mm or a 14 mm x 20 mm balloon) inserted into the hepatic vein via jugular vein access. Simultaneously, the transcutaneous therapeutic ultrasound, either H114H or XDR106-5, was applied to induce cavitation in the liver using a pulsed US treatment. Blood was collected at 24 hours and assessed for alanine and aspartate aminotransferase levels. Animals were sacrificed on day one, and liver tissue was harvested and divided into 1 cm x 1 cm sections and subjected to luciferase and Bradford assays. Representative sections were selected from each liver lobe and subjected to H&E staining and reviewed by a certified pathologist. Hemophilia A dogs tend to be larger and therefore have larger blood vessels, so we tested a new balloon catheter in a normal canine of comparable size (22.5 kg). The 12 mm x 20 mm balloon catheter was used in lobe 2, while the 14 mm x 20 mm balloon catheter was used in lobe 5. Lobe 5 demonstrated high expression (Figure 1A) and had significantly higher luciferase expression compared to lobe 2 (Figure 1B). Liver histology showed little change in the liver on day one following the procedure, with no significant injury noted. ALT and AST values were elevated, but at a comparable level to canines from previous experiments, and no veterinary concern was noted. Preliminary data from pig experiments showed that the H114H and XDR106-5 transducers induced similar levels of luciferase expression (RLU/mg protein) in individual sections. However, the XDR106-5 transducer allowed a much larger area of the lobe to be treated. This led to a tenfold increase in average RLU/mg protein per lobe: 2089 RLU/mg on average when treated with H114H and 25486 RLU/mg when treated with XDR106-5 (Figure 1C). We are able to successfully optimize the transcutaneous UMGD procedure in canines by adjusting the surgical procedure for larger canines, and by incorporating a new ultrasound transducer which effectively treats a larger proportion of a targeted lobe.



109. Assembling Several mRNA Strands for Facilitating mRNA Delivery with and without Using Carriers

Satoshi Uchida[1,2], Naoto Yoshinaga[3], Kyoko Koji[3], Horacio Cabral[3]

[1]Kyoto Prefectural University of Medicine, Kyoto, Japan,[2]Innovation Center of NanoMedicine (iCONM), Kawasaki Institute of Industrial Promotion, Kawasaki, Japan,[3]The University of Tokyo, Tokyo, Japan

For developing messenger RNA (mRNA) delivery carriers, it is still challenging to prevent enzymatic degradation before reaching target cells. Although complicated design of polymers or lipids has contributed to solve this issue to some extent, use of such novel materials requires tremendous processes before clinical approval. Herein, we propose a simple strategy of modulating mRNA steric structure that improves nuclease resistance and delivery efficiency of naked mRNA and existing mRNA carriers. To prevent nuclease attack to mRNA by steric hindrance, we structured mRNA by preparing nanoassemblies (NAs) from several mRNA strands, which were crosslinked with each other using RNA crosslinkers hybridizing to mRNA. NAs thus prepared had about 8 mRNA strands and diameter below 100 nm on average. NAs showed around 100-fold increase in nuclease stability compared to naïve mRNA in their naked form. Further mechanistic studies of NAs indicated the contribution of their high-order structure, as well as secondary structure, on the improvement of nuclease stability. Interestingly, NAs retained their translational activity despite their high-order structure, which prevented nuclease attack. Förster resonance energy transfer-based observation of NA structure in intracellular environment indicates that NAs were dissociated selectively in intracellular environment through endogenous RNA unwinding mechanisms coupled with 5′ cap dependent translation. Ultimately, NAs improved mRNA introduction efficiency in their naked form after transfection to culture cells and to mouse brain. Next, we studied the potential of NAs to improve functionality of an existing mRNA carrier. Polymeric micelles (PMs) from poly(ethylene glycol) (PEG)-polylysine with a core-shell structure of PEG shell and mRNAcontaining core were selected because polylysine is widely used for

complexing nucleic acids. Furthermore, PMs loading plasmid DNA were used in a clinical trial. Both PMs loading naïve mRNA and NAs showed average size below 100 nm, with almost neutral ζ-potential. Further physicochemical evaluations including transmission electron microscopic observation and analytical ultracentrifuge revealed that PMs loading NAs possessed a shell with denser PEG chains and a core with more tightly packaged mRNA compared to PMs loading naïve mRNA. As a result, use of NAs in PMs boosted mRNA nuclease stability in serum and in blood circulation after intravenous injection to mice compared to PMs loading naïve mRNA. Ultimately, PMs loading NAs yielded efficient mRNA introduction after transfection to culture cells and to mouse brain. Importantly, PMs loading NAs were stably distributed to whole brain after injection to cerebrospinal fluid, leading to efficient protein expression in wide area of mouse brain. Notably, the utility of NA formulation in naked and PM-loaded forms was demonstrated using several types of mRNA. Conclusively, our strategy effectively improves the functionalities of naked mRNA and existing mRNA carriers, just by changing mRNA structure without the use of additional materials, providing a versatile platform for future clinical applications.

110. Development of Hydrodynamics-Based

Gene Therapy for Liver Cancer

Kenya Kamimura[1], Takeshi Yokoo[1], Hiroyuki Abe[1], Masato Ohtsuka[2], Hiromi Miura[2], Hiroshi Nishina[3], Shuji Terai[1]

[1]Niigata University, Niigata, Japan,[2]Tokai University, Isehara, Japan,[3]Tokyo Medical and Dental University, Tokyo, Japan

Liver cancer is one of the leading causes of cancer-related deaths worldwide. The primary liver cancer is mainly hepatocellular carcinoma (HCC) and currently available therapeutic options, including embolization and chemotherapy, are not effective against advanced-stage liver cancer due to its heterogeneity, high risk of recurrence, and drug resistance. Although recent developments in the field of molecular-based strategies shed light on chemotherapy for HCC using molecular targeting agents, these agents also have limitations due to the heterogeneity of HCC. Therefore, it is essential to develop a novel therapy, and in this context, we evaluated the gene therapy for HCC using a diphtheria toxin fragment A (DTA) geneexpressing plasmid using a nonviral hydrodynamics-based procedure in this study. The complementary DNA of DTA was inserted into the pIRES2 plasmid vector containing an internal ribosome entry site. The expression of DTA was regulated by a chicken β-actin promoter and a cytomegalovirus enhancer (pCAG-DTA) or the human alphafetoprotein (AFP) promoter (pAFP-DTA). The antitumor effect of DTA expression in HCC cell lines including HLE, HLF, and Huh7 and AFP promoter selectivity was evaluated *in vitro* by examining HCC cell growth. We then examined the *in vivo* effect and safety of the AFP promoter-selective DTA expression using an HCC mouse model established by the hydrodynamic gene delivery of the yes-associated protein (YAP)-expressing plasmid. Mice with the YAP-expressing plasmid demonstrated an HCC incidence of >80% and expression of AFP protein. First, we confirmed the effect of DTA on the inhibition of protein synthesis by the cells based on the disappearance of tdTomato protein expression and GFP protein expression cotransfected upon the delivery of the DTA plasmid. The proliferation of HCC cell lines was

significantly inhibited to <20% upon DTA expression in HCC cells in an AFP promoter-selective manner. In addition, concentration of AFP in the cell culture medium was significantly inhibited by pAFP-DTA transfection. Moreover, a significant inhibition of HCC incidence of 80% to 10%; and a suppression of the AFP tumor marker from 13,448.2 ± 8787.2 to 193.5 ± 129.5 ng/mL and des-gamma-carboxy prothrombin from 590.6 ± 306.7 to 70.4 ± 37.7 ng/mL were evidenced in mice treated with hydrodynamic gene delivery of DTA within 2 months after YAP gene delivery. Serum biochemical factors revealed improvement of hepatobiliary enzymes after the DTA gene therapy due to the shrinkage of the tumor, and no adverse events were observed. These results indicate, for the first time, the effect and safety of hydrodynamics-based gene delivery of DTA for HCC in an *in vivo* mouse model.

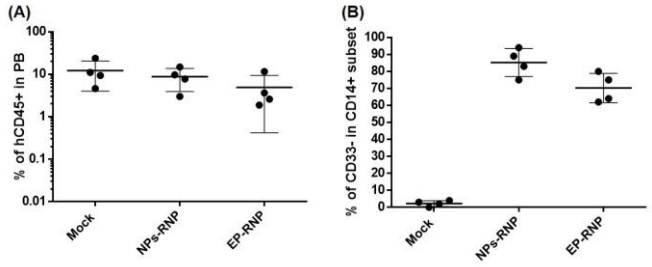
111. Hematopoietic Stem and Progenitor Cells-Targeted Polymeric Nanoparticles for In Vivo Gene Therapy

Rkia El Kharrag[1], Kurt Berckmueller[1], Margaret Cui[1], Ravishankar Madhu[1], Anai Perez[1], Olivier Humbert[1], Hans-Peter Kiem[1,2], Stefan Radtke[1]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA,[2]Department of Medicine, University of Washington School of Medicine, Seattle, WA

Autologous hematopoietic stem and progenitor cell (HSPC) gene therapy has the potential to permanently cure millions of patients suffering from hematological diseases and disorders. However, current approaches depend on expensive specialized cleanrooms for the manipulation of HSPCs *ex vivo* limiting the feasibility and routine clinical application. Consequently, HSPC gene therapy remains inaccessible to many patients especially in infrastructure poor regions. HSPCs-targeted *in vivo* delivery of gene therapy agents could overcome this bottleneck enhancing the portability of gene therapy by avoiding expensive infrastructure. With the aim to perform HSPCs-targeted gene therapy *in vivo*, we adapted a biodegradable poly (β-amino ester) (PBAE)-based nanoparticle (NP) formulation with targeting ability for the modification of HSPCs. We benchmarked the NP-based delivery approach against our existing electroporation (EP)-mediated CRISPR/Cas9 gene-editing strategy to knockout CD33 on HSPCs and protect them from anti-CD33 targeted acute myeloid leukemia immunotherapy. NPs were generated with Cas9 ribonucleoprotein (RNP) complexes and loaded NPs evaluated for size and charge to correlate physiochemical properties with the outcome, and establish quality control standards. NPs and EP gene-edited $CD34^+$ cells were evaluated phenotypically by flow cytometry and functionally in colony-forming cell (CFC) assays, and in NSG xenograft model. The optimal characteristics for RNP-loaded NPs were determined at 150-250 nm and 25-35 mV. Physiochemical assessment of RNP-loaded NP formations provided an upfront quality control of RNP components detecting degraded components significantly and enhancing the predictability and success of editing. NPs achieved more than 85% CD33 knockout using 3-fold lower dose of CRISPR nucleases compared to EP. No impact on erythromyeloid differentiation potential of gene-edited cells in CFC assays was observed. Most importantly, NP-modified CD34+ cells showed efficient and sustained gene editing in *vivo* with improved long-term multilineage engraftment potential in peripheral blood (PB) and bone marrow stem cells in comparison to EP-edited cells **(Fig. A,B)**. Here, we show that PBAE-NPs enable efficient CRISPR/Cas9 gene editing of human CD34+ cells without compromising the viability and long-term multilineage engraftment of human HSPC *in vivo*. Most importantly, we defined physiochemical properties of PBAE-NPs that enable us to not only determine the integrity of our gene-editing agents, but also predict the efficiency on long-term engrafting HSPCs. The requirement of 3-fold less reagents compared to EP, the ability to lyophilize quality-controlled and ready to administer gene therapy reagents, and the opportunity to engineer the surface of PBAE-NPs with HSPC targeting molecules are the technological foundation for feasible, portable, and safe *in vivo* HSPC gene therapy. Ease of production and versatility of cargo options will hopefully enable the field of *in vivo* gene therapy to make a significant step towards a more accessible and portable gene therapy platform. **Figure. A)** Human CD45+ engraftment and **B)** CD33 knockout percentage in CD14+

subset in PB of mice transplanted with CD33 gene-edited CD34+ cells at week 14 post transplantation.



112. Delivery of CRISPR/Cas9 for Recovering the Expression of the Endogenous FVIII in Hemophilia A Mice

Chun-Yu Chen[1], Xiaohe Cai[1], Carol H. Miao[1,2]

[1]Immunity & Immunotherapies, Seattle Children's Hospital Research Institute, Seattle, WA,[2]Department of Pediatrics, University of Washington, Seattle, WA

Hemophilia A (HemA) is a bleeding disorder resulting from a deficiency of the X-linked factor VIII (FVIII) gene. Currently HemA patients is routinely infused with FVIII protein 3-4 times per week as prophylactic treatment, which is costly and inconvenient. Gene therapy represents a very promising alternative method to treat HemA patients. Advancement of biocompatible nanoparticle (NP) technology enabled delivery of nucleic acids safely into the targeted organs. In combination of CRISPR/Cas9 technique, *in vivo* gene edition to correct the mutant FVIII and regain the expression of FVIII is feasible. We examined the *in vitro* gene delivery efficiency of DNA encapsulated NPs and *in vivo* FVIII gene editing, respectively. Since FVIII protein is mainly and naturally made in liver sinusoidal endothelial cells, we synthesized NPs that can selectively target endothelial cells. DNA encapsulated NPs was synthesized using nanoprecipitation by the combination of an organic phase containing chondroitin sulfate and sorbitan ester and an aqueous phase containing p2X-GFP plasmid DNA. DNA encapsulation efficiency was examined by DNA electrophoresis. By titrating different DNA:NPs ratios, our results showed that NPs can carry plasmid DNA efficiently at saturating concentrations.

Next, we used HUVEC cells as endothelial cell model to evaluate the transfection efficiency of DNA encapsulated NPs. The transfected cells were analyzed by flow cytometry after transfection using NPs carrying p2X-GFP plasmid. GFP expression was detected in ~26% of the cells, indicating NPs/DNA can successfully transfect HUVEC cells. Next, we evaluated the efficacy of different sgRNAs for *in vivo* gene editing of mutant FVIII gene by hydrodynamic injection of Cas9/sgRNA plasmid into HemA mice. An immunodeficient hemophilia A (NSG HA) mice that contained premature stop codon in exon 1 of FVIII were used as the HemA mouse model. Two different sgRNAs that can edit wild type FVIII sequence (mF8sgRNA) or mutant FVIII exon 1 sequence (NSGHAsgRNA) were designed, respectively. Efficiency of gene editing was estimated *in vitro* by T7E1 assay using mouse embryonic fibroblast NIH3T3 cells. The results showed that mF8sgRNA can specifically induce double-strand breaks in wild type FVIII gene of NIH3T3 cells. The Cas9/sgRNA plasmids were hydrodynamically injected to NSG HA mice and the FVIII expression was examined by aPTT assay after one week. Both FVIII-targeting sgRNAs can promote the recovery of FVIII expression in NSG HA mice, suggesting the successful gene editing in mutant FVIII. After injection of Cas9/NSGHA sgRNA expression plasmid, FVIII activities maintained at least one month after treatment. Our data showed high transfection efficiency of DNA encapsulated NPs in HUVEC cells. Furthermore, we investigated *in vivo* gene editing using CRISPR/Cas9 technology to correct the mutated FVIII gene and regain the expression of FVIII protein in NSG HA mice. Our future goal is to use NPs that carry Cas9/sgRNA plasmid to correct the mutant FVIII gene in NSG HA mice.

Targeted Gene and Cell Therapy for Cancer

113. Gene-Based Immune Reprogramming Overcomes the Immunosuppressive Microenvironment of Liver Metastases and Enables Protective T Cell Responses

Thomas Kerzel[1], Federica Pedica[2], Stefano Beretta[1], Eloise Scarmadella[1], Tamara Canu[3], Rossana Norata[2], Lucia Sergi Sergi[1], Marco Genua[1], Renato Ostuni[1], Anna Kajaste-Rudnitski[1], Antonio Esposito[3], Masanobu Oshima[4], Francesca Sanvito[1], Mario Leonardo Squadrito[1], Luigi Naldini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]IRCCS San Raffaele Hospital, Milano, Italy,[3]Experimental Imaging Center, San Raffaele Scientific Institute, Milano, Italy,[4]Division of Genetics, Cancer Research Institute,

Kanazawa University, Kanazawa, Japan

The liver hosts an immune suppressive environment, which favors metastatic seeding and proliferation of cancer cells. Current pharmacological treatments, including most recent immunotherapies, fail in the presence of liver metastases (LMS). Therefore, identifying new interventional tools to break tumor tolerance and unleash immune responses in patients with LMS is of

[1] SR-TIGET San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]VitaSalute San Raffaele University, Milano, Italy,[3]German Cancer Research Center

(DKFZ), Heidelberg, Germany

Background: The pleiotropic immunostimulatory effects of cytokines such as interferon alpha (IFN-α) have been extensively investigated in cancer. However, systemic administration leads to sharp oscillations in cytokine plasma levels and OFF-target toxic effects, which strongly

pivotal importance. Our group has shown that vesicular stomatitis virus G protein-

pseudotyped lentiviral vectors (LVs) delivered systemically to mice and non-human primates efficiently target and transduce liver cells, including liver resident macrophages termed Kupffer cells (KCs). Building on these findings, we developed a novel LV-based platform, termed KC-LV, to selectively engineer KCs in vivo with the goal of delivering therapeutic molecules specifically to LMS. To this aim, the KC-LV design exploits a reconstituted mannose receptor c type 1 (MRC1) promoter, active in macrophages, including KCs. To further fine-tune KC specificity, the KC-LV also includes microRNA target (miRT) sequences that prohibit LV transgene expression in liver sinusoidal endothelial cells and hepatocytes. We observed that systemic delivery to mice of a KC-LV driving the fluorescent marker GFP resulted in transgene product expression in KCs, but not in other liver cell types or organs, such as brain, gut, lung, lymph nodes and bone marrow. In mice bearing LMS, GFP expression was enriched in the tissue rim surrounding LMS. To leverage KCLV as a therapeutic platform we included a sequence encoding for interferon a (IFNα), a cytokine with pleotropic immune function. Long term analysis of mice treated systemically with IFNα KC-LV showed a rapidly established, vector dose-dependent and sustained IFNα expression, with no signs of hepatotoxicity, neutropenia or macroscopic skin reactions. IFNα KC-LV systemically delivered to mice challenged with colorectal cancer LMS, either obtained from cancer cell lines or organoids, significantly delayed tumor growth and achieved, in some mice, a complete response. Furthermore, mice that completely cleared LMS were refractory to rechallenge with matched cancer cells indicating persisting adaptive immune protection. Single cell RNA sequencing analysis of LMS revealed upregulation of IFN-responsive genes and altered activation/ polarization profile in tumor infiltrating host cells in mice treated with IFNα KC-LV indicating robust immune reprogramming of the LMS microenvironment. In particular, IFNα KC-LV promoted macrophage skewing from a protumoral (M2-like) to an antitumoral (M1-like) polarization state and expansion of LMS specific CD8 T cells. In summary, we have developed an innovative gene-based platform that upon a single well-tolerated intravenous LV infusion can rapidly establish a protective response against mouse LMS through promotion of macrophage reprograming and adaptive immune activation.

114. Inducible Tumor-Targeted Interferon-α Gene Therapy Inhibits Glioblastoma Multiforme in Mouse Model without Adverse Systemic Effects

Filippo Birocchi[1,2], Melania Cusimano[1], Federico Rossari[1,2], Stefano Beretta[1], Anna Ranghetti[1], Marco Genua[1], Renato Ostuni[1,2], Stefano Colombo[1], Ivan Merelli[1], Paola M. V. Rancoita[1,2], Barbara Costa[3], Angel Peter[3], Nadia Coltella[1], Luigi Naldini[1,2]

limit clinical application. The possibility to specifically target cytokines to the tumor establishing a local source that maintains physiologicalrange concentrations may thus open up new therapeutic perspectives. To this aim, our group has exploited a population of tumor-associated TIE2 expressing macrophages (TEM) to deliver IFN-α specifically to tumors through a cell and gene therapy approach. Transplantation of hematopoietic stem cells (HSCs) transduced with lentiviral vector (LV) expressing *Ifna1* under the control of the *Tie2* promoter induces TEMspecific release of the cytokine in the tumor microenvironment (TME), reprogramming it towards immunological activation.Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor. The unsuccessful results of current therapies make GBM treatment an unmet clinical need. Its strongly immunosuppressive TME and the poor drug delivery across the blood brain barrier, make GBM a suitable candidate for testing our cell-based gene therapy. **Methods:** Mice were transplanted with HSCs transduced with *Tie2-Ifna1* LV. To further improve our platform, we developed an inducible strategy based on fusing destabilizing domains (DD) to the cytokine, thus allowing control of the timing and amount of IFN-α secreted in the TME. Both strategies were tested in a new syngeneic mouse model of GBM, called mGB2, which closely recapitulates several features of the human pathology. **Results:**Upon GBM challenge, mice transplanted with *Tie2-Ifna1* LV transduced HSCs showed remarkable tumor inhibition and improved survival over controls. Notably, a fraction of mice cleared the tumor and survived long term suggesting induction of protective immunity. Similar results were obtained with the inducible platform. The tumor-targeted loco-temporal regulated release of IFN-α induced anti-cancer effect in stringent therapeutic settings, when it was switched ON in already established tumors, and was associated with more effective, durable and safer profile compared to systemic treatment with recombinant IFN-α. Single-cell RNA sequencing and FACS analysis of the tumor infiltrate revealed substantial activation of IFN responsive genes in monocyte/macrophages, B cells and dendritic cells, accompanied by decreased exhaustion of T cells. Moreover, IFN-α gene therapy treatment polarized tumor-associated macrophages (TAMs) towards a pro-inflammatory phenotype, while impairing the recruitment and/or induction of an immunosuppressive subset of TAM, whose gene signature was found to have negative prognostic value in the human disease. **Conclusions:** These results indicate that IFN-α gene therapy can reprogram the GBM TME and stimulate anti-cancer immune response. The promising pre-clinical results obtained so far from our group led to the registration of the first drug based on this strategy, named Temferon®, which is currently in phase I/II clinical trial in our Institute for the treatment of patients with GBM. Our data also demonstrate the feasibility of inducible and intra-tumoral targeted delivery of an immune activating cytokine, thus opening the way to broader application to other cytokines and tumor types.

115. Design and Demonstration of Potent In Vitro and In Vivo Activity for CART-ddBCMA,

a BCMA-Targeted CAR-T Cell Therapy Incorporating a Non-scFv Binding Domain

Janine M. Buonato, Justin Edwards, Liubov Zaritskaya, Ankit Gupta, Laura Richman, David Hilbert, David LaFleur, David Tice
Arcellx, Giathersburg, MD, MD

Chimeric Antigen Receptor (CAR) T cell therapies directed against B-cell maturation antigen (BCMA) have demonstrated compelling clinical activity and manageable safety in subjects with relapsed and refractory Multiple Myeloma (RRMM). Prior reported CAR-T cells have encoded humanized or murine scFvs, or camelid heavy chain antibody fragments with 41BB or CD28 co-stimulatory domains. Herein, we describe the generation and preclinical evaluation of CART-ddBCMA, an anti-BCMA CAR-T cell therapy encoding a non-scFv, synthetic binding domain targeting BCMA with a 4-1BB costimulatory motif and CD3-zeta T cell activation domain. The BCMA binding domain was discovered from a phage display library of D domains based on a ~8 kD highly stable scaffold and engineered for reduced immunogenicity. A human tissue cross-reactivity study using the BCMA binding domain as the test article demonstrated that the binding domain was specific for BCMA. Preclinical in vitro studies of CART-ddBCMA cocultured with BCMA-positive cell lines demonstrated highly potent, dose-dependent measures of cytotoxicity, cytokine production, T cell degranulation, and T cell proliferation. Furthermore, in each assay CART-ddBCMA performed as well as T cells incorporating the BCMA-directed scFv-based C11D5.3 CAR. CART-ddBCMA demonstrated in vivo efficacy in a disseminated BCMA-expressing tumor model in NSG™ immunocompromised mice. Mice engrafted with 5×10^6 U266 cells and grown for 37 days rapidly cleared tumor burden within 7 days following injection of 4.5×10^6, 1.5×10^6, or 0.5×10^6 CART-ddBCMA cells, and tumor clearance was sustained throughout the duration of the experiment. The level of CART-ddBCMA cells detected in circulation 14 days post-T cell transfer was dependent on the initial dose, and at each dose CAR expression was maintained in 90+% of hCD3+ T cells. Based on these promising preclinical data CART-ddBCMA is being studied in a firstin-human phase I clinical study to assess the safety, pharmacokinetics, immunogenicity, efficacy, and duration of effect for patients with RRMM (NCT04155749).

116. A SOX2 Engineered Epigenetic Silencer Factor Represses the Cancer Genetic Program and Eradicate Glioblastoma Development

Vania Broccoli[1], Alessandro Sessa[1], Federica Banfi[1], Valerio Benedetti[1], Mattia Zaghi[1], Luca Massimino[2], Simone Bido[1], Laura Argelich[1], Serena Giannelli[1], Antonio Niro[1]

increase the overall survival. Patients usually undergo tumor resection followed by adjuvant radio- and chemo-therapies that can't prevent recurrences. It has been proposed that some stem cell-like cancer cells (CSCs) with tumor-initiating potential remain in preserved tissue, resist the adjuvant treatments, leading to the re-appearance of the disease. By rational engineering of the SOX2 cancer-associated transcription factor, we generated a synthetic epigenetic repressor named as SOX2 Epigenetic Silencer (SES) that maintains the ability to bind to a large group of its original targets inducing their long-term silencing. By deleting the C-terminal domain of SOX2 and fusing two epigenetic repressor domains, SES is able to effectively inhibit the SOX2 tumorigenic molecular network (rather than activating it as the original TF), including genes crucial for tumor maintenance and growth. By doing this, SES kills both glioma cell lines and patient-derived CSCs both *in vitro* and *in vivo*. Following *in situ*viral transduction of GBM xenografts in mice, we have demonstrated that SES induced a strong growth inhibition of pre-formed tumor of human origin. Remarkably, we have also proved that SES is not harmful to neurons and glia and does not trigger significant transcriptional changes in these cells. Nevertheless, we equipped the vector for our anti-cancer therapy with either a cell-cycle specific promoter or a micro-RNA binding sequence which restricts SES expression exclusively in cancer cells. These data suggest that in situ viral delivery of SES can target the residual cancer cells spread in the tissues and inhibit their growth and survival while leaving unaffected brain parenchyma cells. This approach enables the development of synthetic epigenetic silencers by engineering oncogenic transcription factors that can repress entire tumorigenic transcriptional pathways that are critical for supporting cancer development. Our strategy paves the way to an unprecedented therapeutic opportunity for the treatment of aggressive brain tumors endowed with high levels of efficacy and safety.

117. Oncolytic Adeno-Immunotherapy Expressing IL-12p70 and Immune Checkpoint Blockade PD-L1 Minibody Modulates the Host Immune System to Enable HER2.CAR T-Cells to Cure Pancreatic Tumors

Amanda Rosewell Shaw, Caroline Porter, Tiffany Yip, Way Champ Mah, Katie McKenna, Matthew Dysthe, Robin Parihar, Malcolm Brenner, Masataka Suzuki
Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Oncolytic Adenoviral vectors (OAds) encoding immunomodulatory transgenes ("Armed"OAds) have shown promise as cancer immunotherapy agents. To overcome the limited transgene capacity of OAds, we have combined OAd with a Helper-dependent Ad (HDAd) (CAd system) to locally provide both oncolysis and multiple immunomodulatory molecules encoded by an HDAd. While intratumoral administration of CAd is insufficient to cure metastasized tumors, we have previously demonstrated that combination of local CAd injection with systemic human epidermal growth factor receptor 2 (HER2) specific chimeric antigen receptor T-cells (HER2. CART) provides significant anti-tumor activity in various solid tumor xenograft models including orthotopic metastatic

[1] San Raffaele Scientific Institute, Milano, Italy,[2]Humanitas University, Milano, Italy Glioblastoma multiforme (GBM) is the most common and lethal brain cancer in adults (1-5 cases per 100,000 people per year, 12-15 months of median survival). This is due, in addition to the aggressiveness of the disease, to the inefficacy of current therapies to substantially

model. Based on these encouraging preclinical studies, we proposed this combination immunotherapy with CAd producing IL-12p70, PD-L1

blocking antibody, and an HSVtk safety switch (CAd*Trio*) to treat patients with HER2 positive solid malignancies, including pancreatic adenocarcinoma (PDAC) because high expression levels of HER2 have been associated with poor prognosis in PDAC patients. Since treatment with single immunotherapies (checkpoint inhibitor, oncolytic virus, CAR T-cell) showed limited anti-tumor efficacy in PDAC patients, in this study we evaluated whether our combination immunotherapy enables control of PDAC growth in multiple PDAC models including humanized mice. Combinatorial treatment with CAd*Trio* and HER2. CART cures PDAC tumors within 25 days without tumor recurrence in xenograft models. HER2.CART is the dominant contributor of our combination therapy to the anti-tumor activity in xenograft PDAC models. However, in one tumor model, CAd*Trio* enhanced early HER2.CART infiltration/expansion at the tumor site, leading to better overall anti-tumor activity than treatment with HER2.CART alone. To address how PDAC tumor microenvironment contributes to our immunotherapy, we evaluated our immunotherapies in humanized mouse models. We found that intratumoral CAd*Trio* treatment induced type I IFN responses, including chemotaxis, to enable HER2. CART migration to the tumor site resulting in significant tumor control, and long-term complete responses. In contrast to xenograft models, HER2.CART treatment alone could not cure PDAC tumors in humanized mice. Additionally, using an advanced PDAC tumor model in humanized mice, we found that local CAd*Trio* treatment repolarized distant tumor microenvironments, leading to improved HER2.CART anti-tumor activity at a metastatic tumor site. Elucidation of immune gene signatures indicate CAd*Trio*-dependent stimulation of NK cells, together with *in vitro* data showing CAd*Trio* components and type I IFN induced by CAd*Trio* enhance NK cell anti-PDAC activity, suggest that host immune cells activated through our immunotherapy contribute to the overall anti-tumor effect of our combinatorial treatment. Our data demonstrate that CAd*Trio* and HER2.CART provide complementary activities to eliminate PDAC tumors and may represent a promising therapy for PDAC patients. We have initiated a Phase I clinical trial at BCM (NCT03740256) that has recruited its first patients to test CAd*Trio* with HER2.CART in patients with HER2positive solid tumors including PDAC.

118. UCARTCS1A, an Allogeneic CAR T-Cell Therapy Targeting CS1 in Patients with Relapsed/Refractory Multiple Myeloma (RRMM): Preliminary Translational Results from a First-in-Human Phase I Trial (MELANI-01)

Krina Patel[1], Mini Bharathan[2], Francisco J. Esteva[2], David Siegel[3], Adriana Rossi[4], Mark G. Frattini[2], Julianne Smith[5], Carrie Brownstein[2]

[1] Lymphoma Myeloma, UT MD Anderson Cancer Center, Houston, TX,[2]Cellectis, Inc., New York, NY,[3]John Theurer Cancer Center, Hackensack, NJ,[4]Weill Cornell Medical College, New York, NY,[5]Cellectis, S.A., Paris, France

MELANI-01 (NCT04142619) is a phase I dose-escalation trial of UCARTCS1A, an allogeneic chimeric antigen receptor (CAR) T-cell therapy targeting the CD2 subset-1 (CS1) antigen in patients (pts) with RRMM. UCARTCS1A production includes knockout of the T-cell receptor (to minimize the risk of GVHD) and CS1 (to avoid fratricide during manufacturing). After lymphodepletion with fludarabine/cyclophosphamide, 5 pts received UCARTCS1A: 4 pts in dose level 1 (DL1) (1×10^6 cells/kg) and 1 pt in DL2 (3×10^6 cells/kg). Clinical response per IMWG criteria and exploratory correlates including CS1 expression on MM cells, UCARTCS1A expansion and persistence, changes in serum biomarkers (cytokines, ferritin, liver enzymes), and host immune cell reconstitution were assessed. In all pts, CS1 was expressed in >97% of detected plasma cells. After administration, UCARTCS1A cells were detected in 3/5 pts, with expansion observed in 1 DL1 pt and 1 DL2 pt. In the DL1 pt (4 prior lines of MM therapy), peak UCARTCS1A level in whole blood by flow cytometry was 78 cells/µL and vector copy number (VCN) was 27,385 copies/µg DNA at day (d) 28. This pt achieved an MRD-negative partial response (PR) at d28 and a very good PR (VGPR) by month (mo) 3. Serum lambda light chains (LC) decreased from 503.2 to <1.3 mg/L and M protein from 2.9 to 0.1 g/dL between d0 and mo 3, with LC detectable only by immunofixation at mo 3. Notable clinical findings included grade (G) 2 cytokine release syndrome (CRS), CAR-related hemophagocytic lymphohistiocytosis (HLH), and elevated serum biomarkers including IL-6, IL-8, and IFNγ. This pt died on d109 from organizing pneumonia in the context of prolonged severe lymphopenia in the absence of myeloma progression. In the DL2 pt (13 prior lines of MM therapy), peak UCARTCS1A level was 42 cells/µL (d11) and VCN was 141,090 copies/µg DNA on d9. Serum M protein and kappa LC decreased from 0.9 to 0.4 g/dL and from 15.4 to 1.4 mg/L, respectively, from d0 to d14. The pt achieved a PR at d14. Notable clinical findings included G4 CRS. This pt died on d25 as a result of G5 hemorrhagic pancreatitis in the context of CRS, CAR-related HLH, disseminated mucormycosis, and pseudomonal pneumonia. Among the 3 nonresponding pts in DL1 (11 to 15 prior lines of MM therapy), 1 pt maintained stable disease and 2 pts experienced disease progression. UCARTCS1A cell expansion was associated with meaningful clinical responses, though with notable toxicity including G2-G4 CRS and G5 events. After a brief clinical hold, the study has resumed with protocol updates to address the potential risk of prolonged lymphopenia and severe CRS. These preliminary results support further investigation of UCARTCS1A in RRMM.

119. A Drug-Regulated Anti-CD33 Chimeric

Antigen Receptor with Potent Anti-AML Activity and a Reversible On-Off Switch

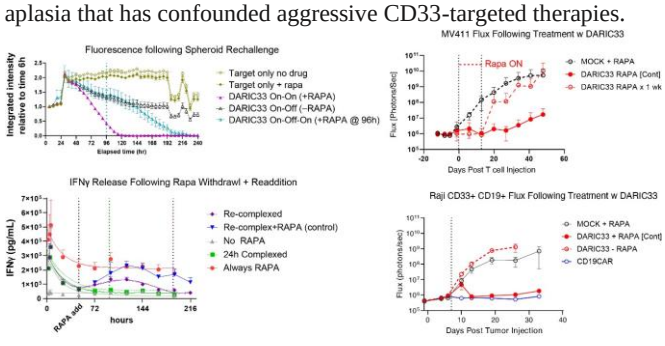
Jacob S. Appelbaum[1,2], Unja Martin[3], Joy Zhang[3], Kaori Oda[1], Mark Pogson[3], Giacomo Tampella[1], Dong Xia[3], Anne-Rachel Krostag[3], April Price[3], Pauline So[3], WeiHang Leung[3], Alexander Astrakhan[3], Jordan Jarjour[3], Joshua Gustafson[1], Michael Jensen[1]

fused to the rapamycin (RAPA)-dependent dimerization domains FRB* and FKBP12. RAPA heterodimerizes antigen binding and signaling receptors, reconstituting CD33-dependent T cell activation. Here, we probe pharmacologic control of DARIC33 T cells using systems for pulsatile RAPA dosing. We find that DARIC33 T cells show nearly complete RAPA-dependent anti-CD33 activity and in vivo efficacy comparable to a clinically validated CD19 CAR. **Results:** DARIC33 T cells preactivated with RAPA for 24hr were washed and cultured in RAPA-free media for different periods prior to stimulation with CD33+ target cells in vitro. Pre-activated DARIC33 T cells showed high levels of cytokine release followed by a progressive decline to baseline levels. Re-addition of RAPA restored antigendependent cytokine release to levels of DARIC33 T cells maintained in RAPA continuously. We also found DARIC33 T cells efficiently eliminate CD33+ spheroids in the presence of RAPA. Following spheroid challenge, DARIC33 T cells were isolated and cultured in RAPA-free media for 48 hours, followed by rechallenge with a second spheroid target. DARIC33 T cells exhibited significantly reduced cytotoxicity after RAPA wash out. However, re-addition of RAPA rescued T cell cytotoxicity, leading to complete spheroid clearance. Thus two in vitro systems show DARIC33 T cell activation by RAPA and reduced cytokine release or cytotoxicity following RAPA withdrawal. In both systems, depressed effector function of DARIC33 T cells following RAPA withdrawal can be restored through re-addition of RAPA. To assess RAPA modulation of DARIC33 activity in vivo, NSG mice engrafted with CD33+ MV4-11 AML cells were treated with DARIC33 T cells + various durations of RAPA. Mice treated continuously with RAPA cleared tumors, whereas mice treated for only 1 week showed tumor regression followed by relapse, confirming RAPA withdrawal deactivates DARIC33 T cells. Next, Raji lymphoma cells, engineered to express CD33 at levels similar to CD19, were injected into NSG mice. The engrafted CD33+CD19+ Raji tumors were then treated with DARIC33 T cells or control CD19 CAR T cells in the presence or absence of RAPA. Mice treated with 10e6 CD19 CAR T cells or 30e6 DARIC33 T cells + RAPA controlled Raji tumor growth. Anti-tumor activity was not observed in mice treated with 30e6 DARIC33 T cells in the absence of RAPA. **Conclusions:** These data demonstrate that DARIC33 T cells exert potent anti-AML activity that can be modulated with RAPA. The resulting temporal control of T cell activation may enable

[1] Seattle Children's Therapeutics, Seattle, WA,[2]Hematology, University of Washington, Seattle, WA,[3]bluebird, bio, Seattle, WA

Background: Acute myeloid leukemia (AML) antigens are expressed on normal hematopoietic precursors. Regulated CAR T cells are an attractive strategy for achieving (1) therapeutic efficacy, (2) hematopoietic recovery, and (3) exhaustion avoidance by temporally constraining antigen-dependent activation. We previously described dimerizing agent regulated immunoreceptor complex targeting CD33 (DARIC33), wherein antigen binding and T cell signaling domains of a chimeric antigen receptor are spatially separated and

optimization of durable effector function, and avoid long-term aplasia that has confounded aggressive CD33-targeted therapies.



Upstream Process Development for AAV Vector Production

120. Co-Identification and Characterization of Host and Viral Protein Interactomes during AAV Production by Two Different Proximity Labeling Methods

Ji Sun Lee, Guangping Gao*, Jun Xie*

Horae Gene Therapy Center, The University of Massachusetts Medical School, Worcester, MA

Scalable manufacturing of high titer and high potency vectors is a challenge for translating recombinant Adeno-Associated Virus (rAAV)-based gene therapeutics into clinical applications. Efficiencies in AAV genome replication, capsid assembly and genome encapsidation are the keys to the success of vector production. Understanding the interactomes in producer cells during AAV vector production will provide insights into developing more efficient production platforms. The proximity-dependent biotin identification method allows detecting protein-protein interactions with a promiscuous biotin ligase fused to a bait protein. These biotinylated proteins can be selectively isolated by biotin-streptavidin capture and identified by mass spectrometry. We grafted two types of biotin ligase, BioID2 or TurboID onto the surface of AAV2 capsid. BioID2 is a smaller biotin ligase and the major disadvantage is its slow kinetics, which necessitates labeling with biotin for 18-24 hours. By contrast, TurboID is greater in size but enable the labeling in ten minutes. Both engineered AAV2 capsids can biotinylate un-biotinylated substrates just like a purified biotin ligase. During AAV vector production by using 293 cell transient transfection method, biotin and ATP were added to the culture medium. Seventytwo hours post transfection, proteins were then selectively isolated by streptavidin-coated beads for mass spectrometry analysis. Rep proteins were found biotinylated regardless of the presence of packageable genomes, suggesting the formation of Rep-Capsid complex before genome encapsidation.

This pre-encapsidation complexes were also previously detected by coimmunoprecipitation, co-sedimentation, and yeast two-hybrid analyses (Ralf Dubielzig, et al., 1999). In addition, a total of 61 cellular proteins were co-identified by both BioID2 and TurboID technologies as direct or indirect interactors with viral proteins possibly involved in vector genome replication, capsid assembly, and genome encapsidation. The main predicted biological functions of the 61 cellular coding genes are associated with cell cycle, immune response, and cell growth by GO term analysis. Ongoing studies aim to further validate contributions of those cellular genes to AAV production process. Our findings may shed some lights to developing strategies to improve vector empty/full particle ratios and virus genome titer. *Co-corresponding authors.

121. The Effects of ITR Structure and Plasmid Backbone on Plasmid Stability and Yield

Ruofan Wang, Shaw Camphire, David Ho, Christopher Porzondek, Bo Li, Guo-an Wang
Vigene Biosciences, Rockville, MD

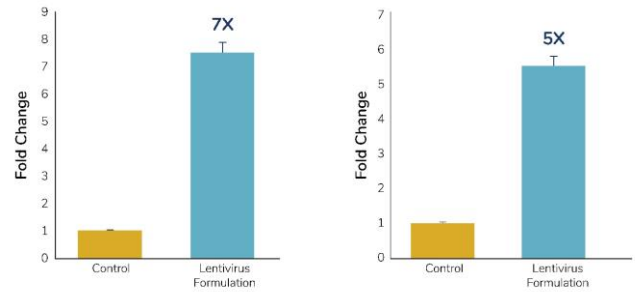
In viral vector production, plasmid integrity is of paramount importance. Plasmid yield is the number one KPI in plasmid

manufacturing, affecting process design, product quality and overall cost. It is common knowledge that AAV ITR-carrying plasmids tend to be unstable, resulting in plasmid variants, extra DNA fragments, and low production yield. Here we present a comprehensive study to document these changes and to understand the causes of ITR instability by means of NGS and restriction analyses. We found that both ITR structure and plasmid backbone contributed to such instability. Our data showed that modification of either ITR structure or plasmid backbone would reduce, and even eliminate, deletions in ITR and result in an increase of plasmid yield from 30% to several folds.

122. Increasing Gene Therapy Vector Production Using Viral Sensitizer Molecules

Jean-Simon Diallo
Virica Biotech, Inc., Ottawa, ON, Canada

Inefficient manufacturing processes can have several important implications during product development. The first and most obvious is cost of goods (COGs), which is driven upwards when GMP compliant material is required. Related are the practical considerations of manufacturing virus using an inefficient process, which means longer times to produce target amounts of virus, more substantial needs in personnel, physical space, and investments in infrastructure. Lastly, there is the issue of maximum feasible dose, where the maximum dose of a manufactured virus given to a patient is limited by the amount of virus that can be produced. Viral Sensitizers (VSETMs) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently overcoming cellular antiviral defenses. VSEs can be used in a range of applications such as improving virus manufacturing yield, improving tumor infection by oncolytic viruses, or transduction of cells by common gene therapy vectors like AAV, adenovirus, and lentivirus. Owing to different molecular mechanisms through which they operate, VSEs can be combined, adapted, and formulated for specific uses. To improve lentivirus production, we employed high throughput screening and DOE methodology to develop a VSE formulation that could improve 3rd generation lentivirus production in adherent HEK293T cells (VSE-LentiTM). We designed a highthroughput method to identify single then multi-VSE compound formulations that enhance lentivirus production. Different transfection reagents, and production scales as well as commonly used lentivirus production enhancers were tested. We found VSE-LentiTM to be compatible with several transfection reagents (PEI Pro, lipofectamine, TransIT lenti). VSE-LentiTM was able to enhance lentivirus production whether added at the time of transfection or up to 24h prior to transfection. Enhancements in lentivirus production were observed over 72 hours, without a requirement to resupply compounds. Improvements in yield observed using VSE-LentiTM were condition-dependent but in pre-optimized conditions lentivirus production conditions, VSE-LentiTM led to >5X increase in TU/ml. **Figure 1. VSE-Lenti™ molecules were added to adherent HEK293T cells at the time of transfection in combination with PEI transfection reagent. Following transfection with luciferase-expressing 3rd generation lentivirus plasmids, a 5-7-fold increase in luciferase activity was observed.)**



123. Transcriptional Response of HEK293 cells to Clinical-Scale Recombinant Adeno Associated Virus Production by Transient Transfection

Cheng-Han Chung[1], Christopher Murphy[1], Douglas

[1] R&D, Lonza Houston Inc, Houston, TX,[2]Process Development, Lonza Houston Inc, Houston, TX

As a leading viral vector for *in vivo* gene therapy, AAV is nonpathogenic and can effectively infect a wide range of human tissues. Currently, most AAV-based gene therapy pipelines rely on AAV manufacturing via transient triple plasmids transfection in human HEK293 cells. However, the productivity of the platform

McCarty[2], Jeffrey Pavlicek[1], Erik Barton[1]
[1]Pharmaceutical Sciences, Bioprocess Research and Development, Gene Therapy Process Development, Pfizer Inc., Morrisville, NC,[2]Rare Disease Research Unit, Pfizer Inc., Morrisville, NC

The Pfizer gene therapy platform relies on transient transfection of HEK293 cells with three plasmids encoding the viral genes required for rAAV replication and assembly. Improving rAAV production is hampered by a lack of fundamental understanding of host cell cofactors and cellular responses to AAV production. We hypothesized that rAAV productivity is constrained by cellular stress or antiviral responses. To investigate potential bottlenecks to productivity, we defined the kinetic transcriptional response of HEK293 cells to rAAV production in a clinical manufacturing-relevant process. We found ~2000 genes differentially expressed after transient transfection and during rAAV production compared with pre-transfection control. Pathways involved in detection of external stimulus, defense response to virus and inflammatory response were regulated at different times post transfection. This indicates that multiplexed cellular responses were triggered by rAAV production, which may negatively impact rAAV productivity. Systematic analyses of the cellular transcriptional response to rAAV production may illuminate genes limiting rAAV yields, thereby enabling the rational design of a next-generation rAAV manufacturing platforms.

124. Vector Engineering of pRep-Cap and pHelper Enhanced AAV Productivity by Triple Transfection in Suspension HEK293 Cells

Caitlin Tripp[1], Jennifer Wang[1], Tsai-Yu Chen[2], Wenling Dong[2], Senthil Ramaswamy[1], Bingnan Gu[1]

AAV for clinical development and commercialization. Previously, we isolated a highly productive clonal cell 5B8 in suspension HEK293, and established a robust cGMP AAV manufacturing platform that was based on triple transfection and supported production in single-use bioreactor up to 250L scale for multiple AAV serotypes. To continue to improve our platform, we designed and evaluated a panel of pRepCap and pHelper plasmid vectors for AAV production. For pRep-Cap vector, we focused on rebalancing expression of Rep and Cap. Through engineering of their native viral promoters, we identified the best combination of Rep-Cap expression levels in pLHI_Rep-Cap [a] that could double the AAV titer across multiple serotypes, e.g. AAV2, AAV5, AAV9, and Anc80L65. The improvement was confirmed with different AAV transgenes in multiple runs in 250L scale. For pHelper vector, we screened five additional candidate helper genes and/or isoforms. Remarkably, we found that the additional co-transfection of one candidate, but not others, with standard triple plasmids could increase the AAV titer 50%-100% over just standard triple transfection method. Consistently, the enhancement was dose-dependent, as more plasmids co-transfection resulted in higher AAV titer up to 100% more. Furthermore, we subcloned the overexpression cassette into the current state-of-the-art vector to create a new generation of pHelper plasmid, named pLHI_Helper [b]. The pLHI_Helper plasmid could be manufactured at the same cost as regular pHelpler but would provide more than 50% productivity compared to the state-of-the-art vector. Taken together, our study suggested that optimization of vector design in AAV manufacturing plasmids is feasible and can significantly improve the AAV productivity in large scale by triple transfection in suspension HEK293 cells. [a, b] Patent pending.

125. High Titer rAAV Production upon Upstream Process Development of Stable Helper-Virus Free ELEVECTA® Producer Cells

Juliana Coronel, Aishwarya Patil, Ahmad Al-Dali, Tom Braß, Helmut Kewes, Christian Niehus, Jens Wölfel, Kerstin Hein, Nicole Faust, Silke Wissing
Cevec Pharmaceuticals GmbH, Cologne, Germany

In recent years, the number of gene therapy products in the biopharma clinical pipeline have increased, as well as the need for scalable manufacturing processes for viral vector production. Recombinant adeno-associated virus (rAAV) is widely used as viral gene therapy vector, however the delivery of the required amounts of AAV-vector particles is still a challenge. We have recently developed a stable helpervirus free rAAV production platform named ELEVECTA®. This AAV production platform consists of mammalian suspension cells which have stably integrated all components necessary to produce AAV, namely the adenovirus helper functions E2A, E4ORF6, VA RNA, as well as AAV replicase, AAV capsid and the gene of interest (GOI) flanked with the AAV ITRs. Production in this system is initiated by induction via doxycycline. Stable rAAV production using the ELEVECTA platform has been proven with different serotypes as well as different GOIs. To further develop the production process using the ELEVECTA® production platform, upstream process optimization was performed with one of CEVEC's AAV8 proof of concept single cell clones. The optimized upstream process, developed in the ambr® 15 was first scaled to 10 L, then in a collaboration with Pall Corporation it was successfully scaled-up to 200 L using the Allegro™ single-use stirred tank bioreactor. A major advantage of fully stable AAV producer cell lines is that this allows for thorough upstream process development. In order to deploy an intensified large-scale process for rAAV production, we applied ATF perfusion technology. The ATF-based perfusion set-up at lab-scale consisted of a stirred tank bioreactor connected to an ATF unit. The cells were inoculated in chemically defined medium free of animal-derived components and cultivated in standard conditions. After reaching the target viable cell density, rAAV production was induced. The harvest was done from the whole cell suspension 4 to 5 days post-induction. Noteworthy, this ATF perfusion process resulted in very high titers (E15 vg/L) and high percentage of full particles (35-40 %).

needs to be improved in order to meet the growing demand of recombinant

126. Genome-Wide CRISPR Activation Screen Reveals That SKA2 and ITPRIP Increase AAV Manufacturing via Cell Cycle Modulation

Hyuncheol Lee[1], Christopher Barnes[2], David Ojala[2], David Schaffer[1,2]

[1]California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, Berkeley, CA,[2]Chemical & Biomolecular Engineering, University of California, Berkeley, Berkeley, CA

Introduction and Methods Recombinant adeno-associated viruses (rAAV) are a leading gene delivery platform; however, current rAAV manufacturing methods cannot meet the demands of the field, which will increasingly be a barrier for the clinical development and commercialization of gene therapies. To address this issue, we developed an AAV-specific genome-wide screening strategy to identify gene targets whose upregulation promotes rAAV packaging. Specifically, an AAV vector library carrying Synergistic Activation Mediator (SAM) guide RNA libraries was generated, and HEK293T cells expressing SAM machinery (a dCas9-VP64 fusion with MS2P65-HSF1) were infected with the AAV library for 48 hours to provide time for modulation of target host factor expression. The cells were then transfected with rAAV packaging genes (pAAV2) and pHelper for another 72 hours to package the rAAV with each vector genome. After the iterative rounds of selection, Next Generation Sequencing was used to analyze the AAV genomes and thus the guide RNAs targeting host factors that increased rAAV packaging. **Results** The most enriched guide RNA targeted the spindle and kinetochore associated complex subunit 2 (SKA2) transcript variant 2 and inositol 1, 4, 5-trisphosphate receptor interacting protein (ITPRIP). In the SKA2 or ITPRIP expressing stable cell line, AAV packaging was increased by 2.2-fold and 3.3-fold, respectively. A cell line expressing both SKA2 and ITPRIP increased AAV titer by 3.8-fold. We analyzed multiple mechanistic steps where target gene expression could impact AAV vector production, including transfection, viral gene expression, the cell cycle, and others. We found that AAV vector genome replication in SKA2 or ITPRIP expressing cell line was higher than that in WT cells. In addition, during AAV production ITPRIP expression increases the proportion of cells in the S-phase, when AAV vector genome replication is known to occur. Moreover, EdU incorporation in SKA2 expressing cells showed elevated S-phase synthesis, suggesting SKA2 may influence cell cycle kinetics during rAAV packaging. Consistent with this finding, culturing in high confluency to arrest in the G0 phase antagonized the effect of SKA2 and ITPRIP, indicating the effect of SKA2 and ITPRIP in rAAV packaging is

mediated by cell cycle modulation. Finally, we performed capsid ELISA and immunoblotting using crude lysate samples normalized by same copy number or same protein concentration and showed expression of SKA2 and ITPRIP increased the AAV full/empty capsid ratio, which suggests increased vector genome replication promoted genome loading into virions. **Conclusions** This broad screening strategy offers a new approach to improve rAAV producing cell lines as well as to unveil the host factors relating to virus packaging. Taken together, our genome wide activation screens revealed host factors, SKA2 and ITPRIP, that increased vector genome replication, the full capsid ratio, and consequently AAV production. **Disclosure** C.B., D.S.O., and D.V.S. are inventors on patents related to cell lines for increased production of AAV. D.V.S is a co-founder of 4D Molecular Therapeutics, which develops novel rAAV therapeutic vectors for clinical use.

Presidential Symposium and Top Abstracts

127. Base Editing Rescues Sickle Cell Disease in Human Hematopoietic Stem Cells and in Mice

Jonathan S. Yen[1], Gregory A. Newby[2,3], Kaitly J. Woodard[1], Thiyagaraj Mayuranathan[1], Cicera Lazzarotto[1], Yichao Li[1], Heather Sheppard-Tillman[4], Shaina N. Porter[5], Yu Yao[1], Kalin Mayberry[1], Kelcee A.
Everette[2,3], Yoonjeong Jang[3], Christopher J. Podracky[3], Elizabeth Thaman[6], Christopher Lechauve[1], Akshay Sharma[7], Jordana M. Henderson[8], Michelle F. Richter[2,3],
Kevin T. Zhao[2,3], Shannon M. Miller[2,3], Tina Wang[2,3], Luke W. Koblan[2,3], Anton P. McCaffrey[8], John F. Tisdale[9], Theodosia A. Kalfa[6,10], Shondra M. PruettMiller[5], Shengdar Q. Tsai[1], Mitchell J. Weiss[1], David R.
Liu[2,3]

[1]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN,[2]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute of Harvard and MIT, Cambridge, MA,[3]Department of Chemistry and Chemical Biology and Howard Hughes Medical Institute, Harvard University, Cambridge, MA,[4]Department of Pathology, St. Jude Children's Research Hospital,
Memphis, TN,[5]Cell and Molecular Biology, St. Jude Children's Research Hospital,
Memphis, TN,[6]Division of Hematology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[7]Bone Marrow Transplantation & Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN,[8]TriLink BioTechnologies, San Diego, CA,[9]Molecular and Clinical Hematology Branch, NHLBI/NIDDK/NIH, Bethesda, MD,[10]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

Sickle cell disease (SCD) is a catastrophic disorder caused by a mutation in the *HBB* gene encoding the β-globin subunit of adult hemoglobin. We used a bespoke adenine base editor (ABE8e-NRCH) to convert the SCD allele (HBB^S) to hemoglobin Makassar (HBB^G), a naturally occurring non-pathogenic allele. Delivery of mRNA encoding ABE8eNRCH and an HBB^S-targeting single guide RNA (sgRNA) into $CD34^+$

hematopoietic stem and progenitor cells (HSPCs) from patients with SCD using a clinical electroporation method resulted in 80% conversion of *HBB*^{S} to *HBB*^{G} with no perturbation of erythropoiesis. Reticulocytes from edited CD34$^+$ cells showed an 80% shift of total β-like globin protein from sickle (β^S) to Makassar (β^G), and a 3-fold decrease in hypoxia-induced sickling. Sixteen weeks after transfer of edited human CD34$^+$ cells into adult immunodeficient mice, the *HBB*^{S}-to-*HBB*^{G} editing frequency in engrafted cells was 68%, indicating durable modification of HSCs. Reticulocytes from the bone marrow of engrafted mice showed a shift of 63% of total β-like protein from β^S to β^G, and a 5-fold decrease in hypoxia-induced sickling. Since human red blood cells (RBCs) do not survive in mouse peripheral blood, we also examined the physiological effects of *HBB*^{S} base editing by electroporating ABE8e-NRCH and sgRNA ribonucleoprotein into HSPCs from a mouse harboring human SCD alleles, followed by transplantation into irradiated host mice. Sixteen weeks after transplantation, 56% of *HBB*^{S} alleles were converted to *HBB*^{G} in bone marrow-repopulating donor HSPCs. Circulating RBCs showed a shift of 84% of total β-like protein from β^S to β^G, and a 3-fold decrease in hypoxia-induced sickling. Mice that received base-edited HSPCs showed rescue of hematologic parameters to near-normal levels and reduced splenic pathology compared to mice transplanted with unmodified HSPCs. Secondary transplantation of edited bone marrow confirmed durable phenotypic rescue of long-term hematopoietic stem cells and when performed with different ratios of edited and unedited HSPCs revealed that ≥20% *HBB*^{S}-to-*HBB*^{G} editing is required for phenotypic rescue, confirming that base editing treatment substantially exceeds this threshold. Human HSPCs showed evidence of p53 activation and large DNA deletions or rearrangements following treatment with Cas9 nuclease targeting *BCL11A*, but not following treatment with ABE8e-NRCH targeting *HBB*^{S}. These findings suggest a potential one-time autologous treatment for individuals with SCD that eliminates pathogenic *HBB*^{S}, generates benign *HBB*^{G}, and minimizes undesired consequences of making double-strand DNA breaks.

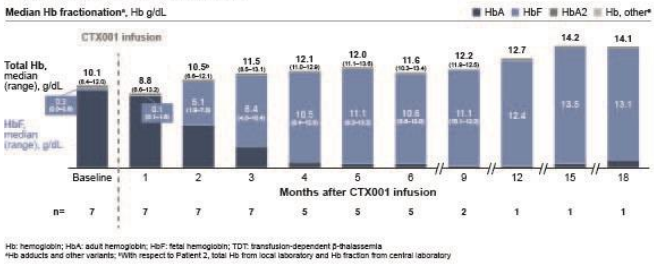
128. Safety and Efficacy Results with a Single Dose of Autologous CRISPR-Cas9-Modified CD34$^+$ Hematopoietic Stem and Progenitor Cells (HSPCs) in Transfusion-Dependent β-Thalassemia (TDT) and Sickle Cell Disease (SCD)

T.W. Ho[1], H. Frangoul[2], Y. Bobruff[1], M.D. Cappellini[3], S. Corbacioglu[4], C.M. Fernandez[1], J. de la Fuente[5], S. Grupp[6], R. Handgretinger[7], S. Imren[8], A. Kattamis[9], J. Lekstrom-Himes[8], F. Locatelli[10], Y. Lu[8], M. Mapara[11], S. Mulcahey[8], M. de Montalembert[12], D. Rondelli[13], N. Shanbhag[8], S. Sheth[14], S. Soni[1], M.H. Steinberg[15], M. Weinstein[1], J. Wu[16], D. Wall[17]

[1]CRISPR Therapeutics, Cambridge, MA,[2]The Children's Hospital, Nashville, TN,[3]University of Milan, Milan, Italy,[4]University of Regensburg, Regensburg, Germany,[5]St Mary's Hospital, London, United Kingdom,[6]University of Pennsylvania, Pennsylvania, PA,[7]University of Tübingen, Tübingen, Germany,[8]Vertex Pharmaceuticals Inc, Boston, MA,[9]University of Athens, Athens, Greece,[10]University of Rome, Rome, Italy,[11]Columbia University, New York, NY,[12]University of Paris, Paris, France,[13]University of Illinois at Chicago, Chicago, IL,[14]Cornell University, New York, NY,[15]Boston University, Boston, MA,[16]University of British Columbia, Vancouver, BC, Canada,[17]University of Toronto, Toronto, ON, Canada

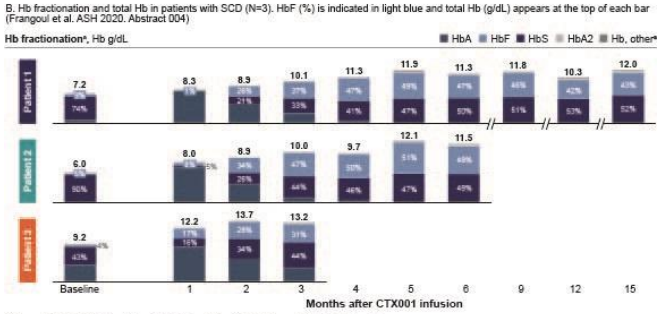
BCL11A is a key transcription factor that suppresses the production of fetal hemoglobin (HbF) in red blood cells (RBCs). In TDT and SCD, elevated HbF is associated with fewer transfusions and clinical complications. To reactivate HbF in RBCs we used the CRISPR-Cas9 platform to edit the erythroid enhancer region of *BCL11A* in HSPCs ex vivo (CTX001™). We present safety and efficacy results from patients (pts) infused with CTX001 with ≥3 months (mo) of follow-up (f/u). CLIMB THAL-111 (TDT, NCT03655678) and CLIMB SCD-121 (SCD, NCT03745287) are multicenter, first-in-human studies of CTX001. Pts aged 12-35 years (ys) with TDT (all genotypes) receiving ≥10 units/y of packed RBC transfusions in the prior 2 ys, and those with severe SCD (≥2 vaso-occlusive crises (VOCs)/y requiring medical care in the prior 2 ys) were eligible. We collected peripheral CD34$^+$ HSPCs by apheresis after mobilization with G-CSF and plerixafor (TDT) or plerixafor alone (SCD). We edited the erythroid enhancer region of *BCL11A* in the enriched CD34$^+$ cells using a specific CRISPR guide-RNA and Cas9 nuclease. Pts received myeloablative busulfan before infusion. We monitored engraftment, AEs, total Hb, HbF, hemolysis, F-cells, RBC transfusions (TDT), and VOCs (SCD). 7 TDT pts (median f/u 8.9 mo, range 3.8-21.5) and 3 SCD pts (median f/u 7.8 mo, 3.8-16.6) received CTX001. Median neutrophil and platelet engraftment were achieved on Day 32 (20-39) and 37 (29-52) respectively in TDT pts, and on Day 22 (17-30) and 30 (30-33) in SCD pts. The safety profile after infusion was generally consistent with myeloablative conditioning and autologous bone marrow transplant. 4 serious AEs (SAEs) related or possibly related to CTX001 occurred in 1 TDT pt in the context of HLH: HLH, headache, ARDS, and IPS. All resolved at time of analysis. The other 9 pts reported no CTX001-related SAEs. Pts received their last transfusion within 2 mo after infusion and showed increases in Hb and HbF over time (Figure). The first pts have been transfusion-free for >20.5 (TDT) and >16.0 (SCD) mo. No SCD pts have had a VOC since infusion. The first SCD pt has been VOC-free for >16.6 mo. CTX001 led to increases in HbF and total Hb in all treated pts. Its post-infusion safety profile is generally consistent with myeloablation. All 7 TDT pts have been transfusion-free for ~2 mo and the 3 SCD pts have had no VOCs. These early data demonstrate CTX001 is a potential functional cure for treatment of TDT and SCD.

A. Median Hb fractionation and total Hb in patients with TDT (N=7). HbF (g/dL) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; TDT: transfusion-dependent β-thalassemia
*Hb adducts and other variants; †With respect to Patient 2, total Hb from local laboratory and Hb fraction from central laboratory

B. Hb fractionation and total Hb in patients with SCD (N=3). HbF (%) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; HbS: sickle hemoglobin; SCD: sickle cell disease
*Hb adducts and other variants

129. Immunostimulatory Bacterial Antigen-Armed Oncolytic Measles Virotherapy Significantly Increases the Potency of Anti-PD1 Checkpoint Therapy

Eleni Panagioti[1,2], Cheyne Kurokawa[1], Kimberly Viker[1], Arun Amayappan[1], Sotiris Sotiriou[3], Kyriakos Chatzopoulos[3], Katayoun Ayasoufi[4], Aaron Johnson[4], Ianko Iankov[1], Evanthia Galanis[1]

was CD8$^+$T cell dependent. Inhibition of the IFN response pathway using the JAK1/JAK2 inhibitor ruxolitinib significantly decreased PDL1 expression on myeloid-derived suppressor cells (MDSCs) in the brain and potentiated the therapeutic effect of MV-s-NAP-uPA and anti-PD1. Our findings support that measles virus strains armed with bacterial immunostimulatory antigens represent an effective strategy to overcome the limited efficacy of immune checkpoint inhibitor based therapies in GBM creating a novel and promising translational strategy for this lethal brain tumor.

130. In-Vivo Engineered B Cells Retain Memory and Secrete High Titers of Anti-HIV Antibodies in Mice

Alessio David Nahmad[1], Cicera Lazzarotto[2], Natalie Zelikson[1], Mary Tenuta[3], Talia Kustin[1], Inbal Reuveni[1], Miri Horovitz-Fried[1], Iris Dotan[1], Rina RosinArbesfeld[1], Adi Stern[1], James Voss[3], Shengdar Tsai[2], Adi Barzel[1]

[1]Tel Aviv University, Tel Aviv, Israel,[2]St Jude Children's Research Hospital, Memphis, TN,[3]The Scripps Research Institute, La Jolla, CA

Eliciting a potent and neutralizing antibody response to diverse and rapidly mutating viruses is a long-standing clinical challenge. HIV specific broadly neutralizing antibodies (bNAbs) can suppress viremia, as demonstrated recently by combination therapy. However, the mean elimination half-life of the bNAbs in patients is shorter than a month, requiring constant administration to prevent the virus from rebounding. Moreover, individuals with prior HIV resistance to the antibodies are excluded from trials and resistance to one antibody occurs when the concentration of the second diminishes. Persistence may be addressed by constitutive expression, however anti-drug antibodies may develop, possibly due to improper glycosylation. B cell engineering provides an opportunity to express a bNAb for adaptive immunity. Both mucosal protection and systemic clearance may be achieved by Class Switch Recombination (CSR). Somatic Hypermutation (SHM) followed by affinity maturation may allow for counteracting viral escape and memory retention allows for increased titers upon viral resurgence. We recently demonstrated that, upon adoptive transfer, bNAb engineered B cells undergo differentiation, memory retention, CSR, SHM and clonal expansion. However, extensive, and expensive *ex-vivo* manipulations hinder clinical potential of this approach. Furthermore, allogeneic B cell therapy necessitates MHC-II compatibility to receive T cell help. To overcome these limitations, we engineer B cells *in-vivo*. In particular, we demonstrate that an injection of two AAV-DJ vectors, one coding for CRISPR/Cas9 and another coding for a bNAb donor cassette, allows for site specific integration in B cells. Following immunizations, we show memory retention and bNAb secretion at high titers. Antibodies secreted by the engineered B cells were found to be of multiple isotypes and IgGs could neutralize autologous and heterologous pseudoviruses. The engineered antibody coding genes underwent somatic hypermutation and clonal selection. Detected engineered cells by flow cytometry included B cells in the blood, plasmablasts and germinal center B cells in the spleen, indicating B lineage differentiation. Biodistribution of the donor AAV over time

[1] Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Neurosurgery, Brigham and Womens Hospital, Harvard Medical School, Boston, MA,[3]Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN,[4]Immunology, Mayo Clinic, Rochester, MN

Clinical immunotherapy approaches are lacking efficacy in the treatment of glioblastoma (GBM). In this study, we sought to reverse local and systemic GBM-induced immunosuppression using the Helicobacter pylori neutrophil-activating protein (NAP), a potent TLR-2 agonist as a novel immunostimulatory transgene expressed in an oncolytic measles virus (MV) platform, retargeted to allow viral entry through the urokinase-type plasminogen activator receptor (uPAR). While single agent murine anti-PD1 treatment or repeat *in situ* immunization with MV-s-NAP-uPA provided modest survival benefit in MV resistant models, the combination treatment led to synergy with a cure rate of 80% in mice bearing intracranial GL261 ($P= 0.047$)and 72% in mice with CT-2A tumors ($P= 0.007$). Combination NAP-immunovirotherapy induced massive influx of lymphoid cells in mouse brain, with CD8$^+$ T cell predominance; therapeutic efficacy

indicated CRISPR-dependent expansion of engineered B cells only in lymphatic tissues. We further assessed the

possible off target effects of our *in vivo* B-cell engineering approach and found limited CRISPR/Cas9 off-target cleavage, using unbiased, highly sensitive, CHANGE-Seq analysis. Finally, we reduced on-target cleavage at undesired tissues by expressing Cas9 from a B cell specific promoter and by coding the gRNA in the donor vector. In summary, we demonstrate that B cells can be safely engineered *in-vivo*. We propose that *in-vivo* B cell engineering should be considered for novel future applications, to address other persistent infections or to treat autoimmune diseases, genetic disorders, and cancer.

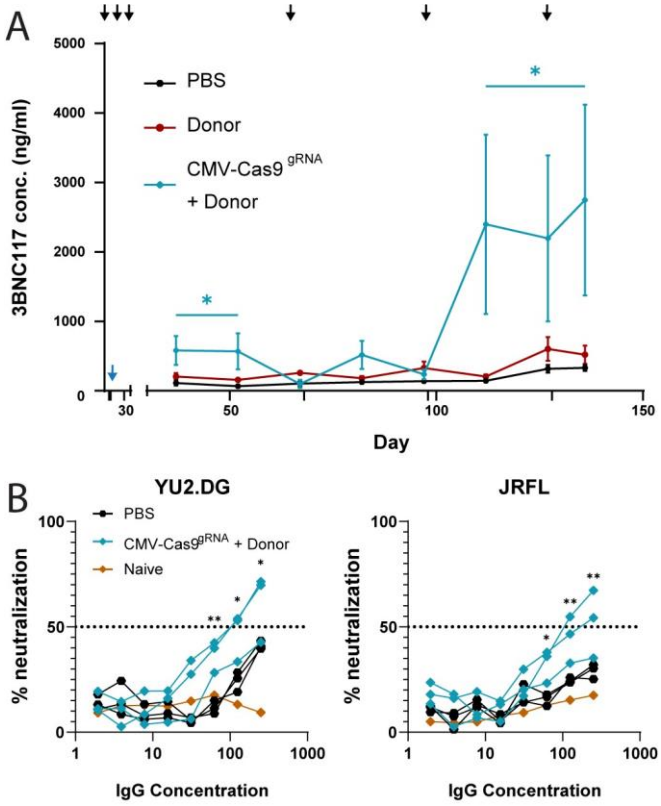


Figure: in-vivo engineered B cells retain memory and secrete high titers of anti-HIV antibodies in mice. A. Serum titers of the antibody integrated in the cells (3BNC117). Black arrows indicate immunizations, blue arrow indicate AAV injection. B. Engineered B cells secrete functional bNAbs. Neutralization of autologous (YU2.DG, left) and heterologous (JRFL, right) pseudoviruses using purified IgGs from last time point as in A.

AAV Therapies for Neurological and Sensory Diseases

131. AAV-Mediated GJB2 Gene Therapy Rescues Hearing Loss and Cochlear Damage in a Mouse Model of Congenital Hearing Loss Caused by Conditional Connexin26 Knockout

Pranav D. Mathur[1], Phillip Uribe[1], Stephanie Szobota[1], Jeremy Barden[1], Sairey Siegel[1], Christopher Bartolome[2], Xiaobo Wang[1], David Jaramillo[2], Anne Harrop-Jones[1], Rayne Fernandez[1], James Vestal[1], Rodrigo Pastenes[1], Bonnie Jacques[1], Steven Pennock[2], Adrian Timmers[2], Fabrice Piu[1], Mark Shearman[2], Alan C Foster[1]
[1]Otonomy Inc., San Diego, CA,[2]AGTC, Cambridge, MA

According to the NIDCD, 2-3 out of every 1,000 children in the United States are born with some degree of hearing loss, with more than half due to genetic factors. Mutations in the *GJB2* gene which encodes the gap junction protein Connexin 26 (CX26) are the most common forms of non-syndromic deafness, responsible for approx. 50% of cases. While in most subjects the onset of hearing loss is prelingual and moderate to severe, in some subjects, hearing loss due to loss of CX26 is mild and progressive. In the inner ear, expression of CX26 is vital for the function of various non-sensory cell types including support cells and fibrocytes. Results from mouse and human studies have revealed that mutations in *Cx26* ultimately lead to near total degeneration of cochlear hair cells. Since the constitutive homozygous *Cx26* knockout is embryonic lethal, we utilized conditional knockouts to study the effect of losing CX26 protein in the cells of the inner ear. We utilized two different conditional knockout strains (*Cx26* cKO) generated by crossing $Cx26^{loxp/loxp}$ mice with either an inducible *cre* mouse line or with a constitutive *cre* mouse line. Using the inducible *cre* line, we knocked out *Cx26* with temporal control and observed varying degrees of hearing loss and cochlear defects dependent on the time of *cre* induction. Early postnatal *cre* induction caused severe to profound hearing loss in the *Cx26* cKO mice when assessed at postnatal day 30, whereas later induction of *cre* resulted in mild to moderate hearing loss that was progressive in nature. Constitutive *cre Cx26* cKO animals, by virtue of embryonic *cre* expression in the inner ear tissues, displayed severe to profound hearing loss across all frequencies tested. The availability of these various mouse models enabled us to evaluate AAV-mediated *GJB2* gene therapy across a spectrum of hearing loss severity that mimics known human phenotypes. We previously reported the identification of novel adeno-associated viral (AAV) vectors that produce efficient expression of a gene of interest in cochlear support cells in rodents and non-human primates. We designed an AAV vector with an optimized capsid, promoter and human *GJB2* gene elements (OTO-825) that provides excellent expression of CX26 in cochlear support cells and fibrocytes. We also generated an identical AAV vector that expresses CX26 with a FLAG-tag to allow identification of virally expressed CX26 (OTO-825-FLAG). In cell-based assays, utilizing HeLa cells that do not normally express CX26, both OTO-825 and OTO-825-FLAG induced expression of CX26 that was correctly trafficked to the cell membrane. Injection of OTO-825-FLAG into the cochleae of mice provided near total expression of CX26-FLAG in our cells of interest throughout the cochlea (from base to apex). Compared with vehicle, intracochlear administration of OTO-825 to postnatal *Cx26* cKO mice substantially restored CX26 expression and provided a marked improvement in hearing across multiple frequencies. In addition, OTO-825-injected *Cx26* cKO mice had greatly improved cochlear morphology relative to those injected with

vehicle. Sub-cellular localization of the CX26 protein in rescued animals was normal and apparent in support cells throughout the cochlea, and these animals showed increased numbers of surviving hair cells. These preclinical results with AAV-mediated GJB2 gene therapy support the use of OTO-825 as a clinical candidate to treat congenital hearing loss caused by GJB2 deficiency.

132. AAV9 Mediated Delivery of PUF RNA Targeting System Corrects Molecular and Functional Defects in Amyotonic Dystrophy Type 1 Mouse Model

Ranjan Batra[1], Daniela M. Roth[2], Claire Geddes[1], Haydee Gutierrez[1], Nandini Narayan[1], Aaron Berlin[3], Greg Nachtrab[3], Dan Gibbs[3]

[1]R&D, Locanabio, Inc., San Diego, CA,[2]Molecular Biology, Locanabio, Inc., San Diego, CA,[3]Vector Development, Locanabio, Inc., San Diego, CA

Myotonic dystrophy type I (DM1) is a multisystemic autosomaldominant inherited disorder caused by CUG microsatellite repeat expansions (MREs) in the 3' untranslated region (UTR) of the DMPK mRNA. Previously, we showed that a CRISPR-based RNA-targeting gene therapy has the potential to eliminate toxic RNAs expressed from repetitive tracts in DM1 in primary patient cells and a DM1 mouse model. We engineered a novel PUF RNA-binding protein system derived from the naturally-occurring human PUM1 protein linked with an RNA endonuclease (E17) derived from human ZC3H112A to target and cleave expanded DM1-related CUG repeats. AAV9-packaged PUF-E17 was delivered via intramuscular (IM) and intravenous (IV) injections to adult (8-weeks old) HSALR DM1 mice. We report sustained PUF-E17 expression at 4- and 12-weeks post injection. Consequently, we observed ~70% decrease in CUG nuclear foci by RNA-FISH, ~50% decrease in the levels of CUG-containing RNA, and efficient reversal of DM1-related mis-splicing to near wildtype levels 4 weeks post injection. We also compared RNA-targeting Cas9, Cas13d, and PUF-E17 systems in vivo and show comparable efficacy in elimination of toxic CUG repeats. Finally, we show that PUF-E17 treatment is safe in HSALR mice giving us multiple safe treatment options for this neuromuscular disorder.

133. Intracorneal and Sequential Contralateral Dosing of AAV-opt-ARSB Reverses MPS VI Corneal Clouding

Matthew L. Hirsch[1], Jacklyn H. Salmon[2], Liujiang Song[1], Darby Roberts[2], Jacquelyn J. Bower[1], Prabhakar Bastola[1], Brian C. Gilger[2]

[1] Ophthalmology, University of North Carolina, Chapel Hill, NC,[2]Clinical Sciences, North Carolina State University, Raleigh, NC

Mucopolysaccharidosis VI (MPS VI) is a rare, autosomal recessive lysosomal storage disease caused by mutations in ARSB which encodes arylsulfatase B. Corneal blindness in MPS VI patients compromises life quality and there is no effective treatment. The purpose of these studies was to validate an AAV gene therapy for MPS VI corneal clouding. Initially, the human ARSB ORF was compared to codon optimized cDNA sequences to identify the variant most productive for arysulfatase B activity in vitro (termed opt-ARSB). MPS VI felines, homozygous for a null ARSB mutation, presented diffuse mild corneal clouding and peripheral corneal vascularization OU with no retinal dysfunction. At 21 weeks of age, $ARSB^{-/-}$ felines were unilaterally administered AAV8-opt-ARSB ($1e^9$ viral genomes (vg)) via intracorneal injection. Clinical ophthalmic examinations (slit lamp biomicroscopy, tonometry, pachymetry, ophthalmoscopy, corneal OCT) were performed throughout the study and corneal confocal microscopy was employed at the experimental conclusion. Following the AAV8-opt-ARSB injection, the pre-existing corneal clouding cleared in the central 85-95% of the cornea within 3 weeks. In contrast, the severity of corneal disease progressed in the contralateral vehicle treated cornea. Eight weeks following the initial vector injection, the vehicle treated $ARSB^{-/-}$ cornea was administered AAV8-opt-ARSB ($1e^9$vg). Within 2 weeks of this sequential injection clearance of the storage disease was observed. All AAV8-opt-ARSB injections to the corneal stroma were well tolerated and all vector treated $ARSB^{-/-}$ corneas exhibited >85% clarity until the humane endpoint (90 days following the sequential AAV8-opt-ARSB administration). Vector biodistribution analyses, vector derived cDNA abundance, histological analyses, as well as serum AAV8 neutralizing antibody titers will also be presented. The collective data following intracorneal injection of AAV8-opt-ARSB in MPS VI felines, in conjunction with the large data set of a similar approach for MPS I corneal disease, suggest that intracorneal AAV gene therapy is safe and effective to reverse corneal clouding observed in several lysosomal storage diseases. Importantly, this is the first study to demonstrate in any disease model that sequential AAV vector dosing of the cornea, is safe and remarkably therapeutic with no immunological or adverse consequences.

134. Efficacy and Biodistribution of Anc80-RKhRPGRIP1 Gene Therapy in a Mouse Model of Rpgrip1 Deficiency and in Non-Human Primate

Binit Kumar, Lambert Edelmann, Greg Voronin, Anna Mollin, Eun-Hee Park, Eun-Hee Park, Mehdi Doroudchi, Steve DeMarco, Stephen Jung, Hur Dolunay

Kanal, Marla Weetall

PTC Therapeutics Inc, South Plainfield, NJ

Introduction: Mutations in the *RPGRIP1* (retinitis pigmentosa GTPase regulator interacting protein 1) gene are a cause of LCA6 (Leber congenital amaurosis 6) and account for ~3-5% of total autosomal recessive blindness. RPGRIP1 plays a vital role in the development and maintenance of photoreceptors due to its role in the

trafficking of proteins along the connecting cilium. A null mutation in the *RPGRIP1* gene leads to progressive and irreversible blindness typically during childhood. Other mutations are associated with a later onset of disease. We are currently investigating RPGRIP 1 gene therapy in a *Rpgrip1* knockout mouse model. Here, we describe biodistribution studies in wildtype (WT) mice and non-human primates (NHP) and efficacy studies in *Rpgrip1* knockout mice. **Methods:** An initial biodistribution and dose response study was conducted using five doses (between 6.76

x 10^9 - 8.02 x 10^{11} vg/eyetotal dose) in 4-6 week-old WT C57BL/6J mice and analyzed for vector biodistribution using qPCR and protein expression using an ECL (Enhanced Chemiluminescence) assay. The extent of Anc80-hRK-RPGRIP1 transduction (at doses 4.5 x 10^{10} and 4.5 x 10^{11} vg/eye in NHP and 2e9 vg/eye in mouse) was determined using *in situ* hybridization (ISH) probes against RPGRIP1 DNA and mRNA in mouse and NHP. To assess efficacy of RPGRIP1 gene replacement therapy in PND21 *Rpgrip1*$^{-/-}$ mice, an Anc80 AAV vector containing a human rhodopsin kinase promotor driving the expression of RPGRIP1 was injected subretinally into one eye of each *Rpgrip1*$^{-/-}$ mouse at doses of 7.36 x 10^{10}, 2.43 x 10^{11} or 8.02 x 10^{11} vg/eye. Each contralateral eye was injected with vehicle as a control. At 12- and 24-weeks post-subretinal injections, the full field electroretinograms (ERGs) and optical coherence tomography (OCT) were recorded for measuring retinal function and structure, respectively. *Rpgrip1*$^{-/-}$ mice injected subretinally with Anc80-hRK-RPGRIP1 were analyzed for RPGRIP1 protein localization using immunohistochemistry along with other markers, such as acetylated tubulin and rootletin. **Results:** Subretinal delivery of Anc80-hRK-RPGRIP1 in WT C57BL/6J resulted in a dose-dependent increase in AAV vector distribution and human RPGRIP1 protein expression. Similarly, NHP retinas showed a dose dependent increase in Anc80-hRK-RPGRIP1 transduction and mRNA expression analyzed using ISH assay. ERG and OCT analysis showed improved preservation of photoreceptor/retinal function and improved photoreceptor survival in the treated eyes compared to untreated eyes 12 weeks after subretinal injection in *Rpgrip1*$^{-/-}$ mice. Subretinal delivery of the Anc80-hRK-RPGRIP1 in *Rpgrip1*$^{-/-}$ mice confirmed human RPGRIP1 protein was expressed specifically in photoreceptors and localized correctly in the connecting cilia. **Conclusions:** The present study demonstrates improvement in photoreceptor survival and function after subretinal delivery of Anc80-hRK-RPGRIP1. Efficacy was associated with the proper localization of human RPGRIP1 protein at the connecting cilium.

135. Bicistronic AAV Gene Therapy for Tay-Sachs and Sandhoff Diseases

Toloo Taghian[1], Deborah Fernau[1], Kalajan L. Mercado[2], Lauren Ellis[2], Elise Diffie[2], Amanda Gross[2], Anne S. Maguire[2], Ana Rita Batista[1], Stephanie Bertrand[3], Monique Otero[1], Rachel Prestigiacomo[3], Rachael Gately[3], Jillian Gallagher[1], Hannah Lahey[1], Amanda Taylor[2], Jey Koehler[2], Douglas R. Martin[2], Miguel Sena-Esteves[1], Heather L. Gray-Edwards[1]

[1] Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Auburn University, Auburn, AL,[3]Tufts Cummings School of Veterinary Medicine, Grafton, MA

Tay-Sachs and Sandhoff diseases (TSD, SD) are neurodegenerative diseases, with similar clinical manifestations, caused by mutations in alpha or beta subunit of enzyme Hexosaminidase (Hex) respectively. Deficiency of Hex results in GM2 ganglioside storage and neuronal death. The Hex isozyme (Hex A) that degrades GM2 in humans is a heterodimer of alpha and beta subunits; therefore, co-expression of both subunits is necessary for restoration of Hex function. Here we describe the therapeutic efficacy of a single, bicistronic AAV9 vector construct that delivers both Hex subunits simultaneously in a naturally occurring sheep model of TSD. TSD sheep were injected with 1E14 vg total intravenously (IV, n=5, 5 days of age) or via the cerebrospinal fluid (CSF, n=5, 3-6 weeks of age). Sheep treated intravenously survived to 18±5 months of age and CSF treated sheep are all ongoing with the oldest now more that 3 years of age. Untreated TSD sheep reach the humane endpoint at ~ 9 month of age. GM2 ganglioside levels in CSF of 1- and 2-year-old animals treated by CSF administration were completely normalized ($P<0.0009$ vs TSD; $P<0.88$ vs normal), however, the GM2 ganglioside levels in the IV cohort were between that in untreated TSD and normal sheep. Both IV and CSF AAV treated TSD sheep exhibit marked attenuation of neurologic disease as well as normal cognition as measured by maze testing. Magnetic resonance spectroscopy (MRS) of treated sheep thalamus at ~2 years of age showed normalization of markers of neuronal health, myelination and metabolism in the CSF cohort and a slight improvement in neuronal health in the IV cohort. Diffusion tensor imaging (DTI) analysis demonstrates the decrease in microstructural integrity and increase in water diffusion in white matter of TSD brain which is a consistent finding in neurodegenerative diseases. IV treatment partially corrected these changes in some brain regions. IV treatment resulted in Hex A activity in spinal cord at ~ 70% and 100% of normal level in cervical and lumbar sections, ~ 20% in cerebellum and brainstem and lower levels in other brain regions. GM2 content in caudal aspect of the brain correlates (>80%) with Hex A activity. HexA level in skeletal muscle was ~90% of normal, but levels were lower in skeletal and other peripheral organs and tissues. Further studies are ongoing with both cohorts. Non-invasive imaging and GM2 content analysis on CSF have been routinely performed on long term CSF cohort (~ 3 years of age) to monitor the therapeutic efficacy using non-invasive biomarkers of the disease and provide data to further aid clinical translation. To enable early assessments of biomarkers and biochemistry in postmortem tissues after CSF administration, a short-term CSF treatment cohort has been initiated (n=4, endpoint ~6 months of age). These data show promise for a minimally invasive treatment for TSD and SD using this bicistronic

vector construct which will provide the basis for submission of a pre-IND application.

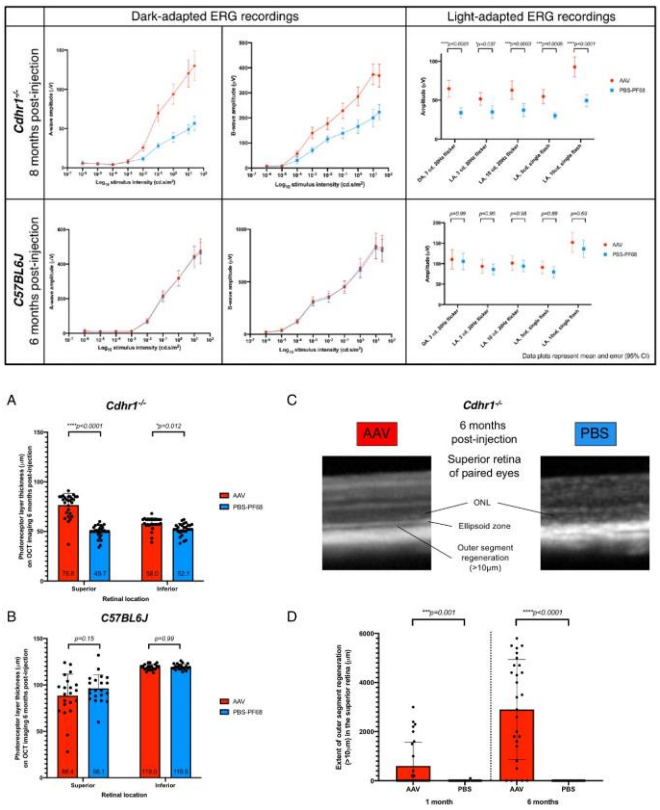
136. Gene Therapy Rescues Cone and Rod Function in a Pre-Clinical Model of *CDHR1*-Associated Retinal Degeneration through Restoration of Photoreceptor Outer Segments

Imran H. Yusuf, Michelle E. McClements, Robert E. MacLaren, Peter Charbel Issa

Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom

Purpose To evaluate the efficacy and safety of retinal gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration - an as yet untreatable, blinding disorder characterised by progressive cone and rod photoreceptor degeneration. **Methods** *Cdhr1*$^{-/-}$ *(n=28)* and *C57BL6J* control mice *(n=23)* underwent paired sub-retinal injections of AAV8.GRK1.CDHR1 ($1.5x10^8$) and PBS vehicle control in the fellow eye at 4 weeks of age. Dark- and light-adapted electroretinography (ERG) was undertaken at 2, 4, 6 and 8 months post-injection. Photoreceptor layer thickness measurements were compared using optical coherence tomography (OCT) imaging at 1 and 6 months post-injection. **Results** In *Cdhr1*$^{-/-}$ mice, sub-retinal delivery of AAV8.GRK1.

CDHR1 rescued A-wave amplitudes (*p<0.0001* at all time points) and B-wave amplitudes (*p<0.0001* from 6 months) on the dark-adapted ERG luminance series when compared with PBS-injected control eyes **(Fig.1)**. Light-adapted flicker ERG amplitudes were greater in AAV-treated eyes at 8-months post-injection (*p<0.0001*). Sub-retinal injection of AAV8.GRK1.CDHR1 preserved photoreceptor layer thickness in the superior retina versus PBS-injected control eyes at 6-months post-injection (mean: 76.6µm versus 49.7µm; *p<0.0001;* **Fig.2**). OCT changes consistent with photoreceptor outer segment regeneration and restoration of the ellipsoid zone were only identified in AAV-treated eyes, with therapeutic effect seen as early as 1-month post-injection *(p=0.001;* **Fig.2***)*. In *C57BL6J* mice, there was no difference in ERG assessments (A-wave, *p=0.65*; B-wave, *p=0.47*; Cone responses, *p=0.09;* **Fig.1**) or photoreceptor thickness measurements at 6 months between AAV and PBS-injected eyes *(p=0.19;* **Fig.2***)*. **Conclusion** These data provide proof-of-principle of the efficacy and safety of *CDHR1* gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration. Rod and cone rescue occur through prevention of photoreceptor cell death and regeneration of photoreceptor outer segments. A follow-on clinical trial in patients with *CDHR1*-associated retinal degeneration is warranted.



AAV Therapies for Neurological and Sensory Diseases

137. scAAV9 Gene Replacement Therapy for Epileptic SLC13A5 Deficiency

Rachel Marion Bailey, Lauren Bailey, Morgan Schackmuth, Irvin Garza

Center for Alzheimer's and Neurodegenerative Diseases, University of Texas Southwestern Medical Center, Dallas, TX

SLC13A5 Deficiency is an autosomal recessive disorder. This severe and rare form of epileptic encephalopathy is due to mutations in the *SLC13A5* gene, which codes for a plasma membrane sodiumdependent citrate transporter. To date all tested mutations result in no or a much reduced amount of the citrate transported inside the cells. Affected children present with seizures beginning within a few days of birth that persist throughout life. They show difficulty with speech production, limited and slow motor progress and many never achieving independent walking. Currently there are no treatments for SLC13A5 deficiency that target the underlying cause of disease and patients require constant supervision and care. Anti-epileptic drugs have varying success even amongst siblings, and children can succumb to complications of the seizures. Gene replacement therapy represents a therapeutic option for SLC13A5 Deficiency. We developed a self-complementary vector encoding a codonoptimized human SLC13A5 gene (scAAV9/SLC13A5), the unaltered design of which could be appropriate for human use. This study aims to compare safety and efficacy of scAAV9/SLC13A5 delivered intrathecally-lumbar puncture (IT) or intra-cisterna magna (ICM) in SLC13A5 knockout (KO) mice and wild type (WT) littermates. KO mice do not have an overt phenotype; however, similarly to patients, they have increased extracellular citrate and

abnormalities in TCA intermediates. KO mice have low, but increased epileptic activity as compared to WT mice and continuous EEG recordings can detect seizure types ranging from myoclonic and focal and generalized convulsive, like those seen in patients. KO mice and WT littermates were treated with scAAV9/SLC13A5 via IT delivery or ICM delivery at 10-12 weeks of age. Mice were monitored for weight and survival. Blood was collected at baseline and then monthly after injection and ~5 months after treatment, mice received telemetry implants to record EEG and EMG activity over two 60 hour recording sessions. These mice were then tested for susceptibility to seizure induction by pentylenetetrazol (PTZ). In agreement with previous studies, at baseline, KO mice had increased plasma citrate levels as compared to WT littermates. At one-month post-treatment and beyond, KO mice treated with scAAV9/SLC13A5 had significantly decreased plasma citrate levels while KO mice treated with vehicle had sustained, high citrate levels. EEG assessments showed that vehicle treated KO mice had increased spike train activity and seizure frequency compared to their WT littermates and, importantly, that vector treatment reduced this epileptic activity with greater rescue achieved with ICM delivery than IT delivery. Using the Racine scale, SLC13A5 KO mice also had increased PTZ-induced seizure susceptibility, which was rescued to WT levels with ICM delivery of scAAV9/SLC13A5 and to a lesser extent with IT delivery. Additionally, the safety profile of the SLC13A5 vector was excellent with no adverse effects on weight, general activity or survival in KO or WT mice with either IT or ICM delivery. Overall, our preclinical results suggest that gene replacement therapy with scAAV9/SLC13A5 could provide a meaningful benefit to SLC13A5 patients.

Advances in Cellular and Immunotherapies?

138. Dissecting the Transcriptional and Epigenetic Landscape of hiPSC-Derived Neural Stem and Progenitor Cells: Implications for Cell Therapy Approaches

Vasco Meneghini[1], Marco Luciani[1], Chiara Garsia[1], Luca
Petiti[2], Ingrid Cifola[3], Stefano Beretta[1], Ivan Merelli[1,3], Clelia Peano[4,5], Annarita Miccio[6], Angela Gritti[1]
[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Computational and Chemical Biology, Fondazione Istituto Italiano di Tecnologia, Genova, Italy,[3]Institute for Biomedical Technologies (ITB), National Research Council (CNR), Segrate, Italy,[4]Institute of Genetic and Biomedical Research, UoS of Milan, National Research Council (CNR), Rozzano, Italy,[5]Genomic Unit, IRCCS Humanitas Clinical and Research
Center, Rozzano, Italy,[6]Laboratory of Chromatin and Gene Regulation during Development, INSERM UMR1163, Imagine Institute, Paris, France

Human induced pluripotent stem cell (hiPSC)-derived neural stem/progenitor cells (hiPS-NSPCs) are a promising cell source for cell therapy approaches and for the development of novel *ex vivo* gene therapy strategies to target neurodegenerative disorders with unmet clinical need. We have shown that intracerebral transplantation of genetically modified hiPS-NSPCs provides full rescue of the enzymatic deficit in the murine model of metachromatic leukodystrophy (MLD), a fatal demyelinating disease caused by genetic mutations of the arylsulfatase A (ARSA) enzyme. The optimization of hiPS-NSPC production (purity, homogeneity) and a comprehensive safety assessment are mandatory in view of prospective clinical application. In this study, we evaluated the transcriptional and epigenetic mechanisms underlying the hiPSC to neural commitment and complemented these data with phenotypic and functional studies in order to define a comprehensive hiPS-NSPC signature and safety profile. Computational integration of RNA-seq and ChIP-seq data revealed a strong downregulation of pathways regulating pluripotency, cell cycle, and cancer-related processes with the concomitant appearance of a distinct "neural signature" in hiPS-NSPCs. Interestingly, we highlighted a dramatic change in the usage of cell-specific enhancers and super-enhancers during hiPSC neural commitment, suggesting its major role in the generation and maintenance of hiPS-NSPCs. Differences in the transcriptomic and epigenetic profiles between hiPS-NSPCs and human fetal NSCs (hfNSC, used as reference) could be ascribed to culture conditions, regionalization pattern, and differentiation potential, with no major signs of abnormal differentiation and activation/misregulation of cancer-related pathways attributable to a pluripotent "memory". Single cell RNA-seq analyses confirmed that hiPS-NSPCs are a heterogeneous cell population mainly composed by radial glial stem cells and committed neuronal and glia progenitors, and do not include pluripotent cells. Indeed, upon intraventricular transplantation in neonatal immunodeficient mice hiPS-NSPCs engrafted and widely dispersed in the brain parenchyma, migrating along the rostro-causal axis and differentiating in mature neurons and glial cells, with no sign of abnormal cell proliferation up to 12 months after treatment.This study contributes to the development of strategies for increasing safety and efficiency of hiPS-NSPC transplantation approaches for the treatment of neurodegenerative and demyelinating disorders.

139. Receptor Engineered TRuC Tregs Maintain a Regulatory Phenotype and Are Suppressive in a Murine Model of Hemophilia A

Jyoti Rana, Sandeep R. P. Kumar, Maite Munoz, Moanaro Biswas
School of Medicine, IUPUI, Indianapolis, IN

The most serious complication to clotting factor VIII (FVIII) replacement therapy in hemophilia remains the development of inhibitory antibodies (inhibitors), which occurs in a significant proportion of patients with severe disease. Tolerance to exogenously administered FVIII is strongly dependent on regulatory T cells (Tregs) and it is expected that "redirecting" antigen specificity by engineering Tregs with synthetic receptors will overcome low inherent precursor frequencies and simultaneously augment suppressive functions in Treg cellular therapy. Our preliminary data indicate that employing a high affinity chimeric antigen receptor

(CAR) molecule paradoxically results in heightened signaling and loss of suppressive activity in transduced Tregs. Therefore, we applied an alternative approach, which is to complex a FVIII antibody based single-chain variable fragment (scFv) to the N-terminus of murine CD3ε subunit of TCR complex. The resulting TCR fusion construct (TRuC) Treg is able to recapitulate TCR based signaling in an MHC-independent fashion. We initially confirmed TCR dependent surface expression of TRuCs by flow cytometry using a FVIII binding assay, where co-transduction of FVIII-TRuC with TCR and CD3 components resulted in transportation of the engineered receptor to the plasma membrane surface in 5KCα^{-}β^{-} cells (which lack TCRα and β) and in human HEK-293 cells (which lack TCR and CD3). FVIII stimulation of TRuC-Tregs *in vitro* led to upregulation of CD69, Ki67, CD28, FoxP3, and a 5-fold increase in CTLA4 expression (p=0.0001, 1-way ANOVA). TRuC Tregs secreted significantly lower levels of cytokines IL-2, IL-4, IL-17, IL-10 and IFNγ as compared to CAR Tregs. This was confirmed by intracellular cytokine staining. Phospho-flow cytometry and western blotting confirmed dampened expression of critical signaling proteins pAKT S473, pERK and pS6 in TRuC Tregs, which was similar to levels observed on triggering the endogenous TCR in Tregs with anti-CD3/28 microbeads. These results confirm that FVIII stimulated TRuC Tregs maintain a Treg phenotype. Importantly, FVIII stimulation and TRuC signaling did not result in a loss of lineage stability in transduced Tregs, as TRuC Tregs from FoxP3-GFP mice retained FoxP3 expression in vivo (92.75±0.3% GFP^{+} cells). We next investigated whether TRuC Treg functional responses was sufficient to maintain a suppressive phenotype *in vitro* and *in vivo*. FVIII TRuC Tregs were able to suppress the *in vitro* proliferation of TRuC Tconv responders when stimulated with low dose (0.1 IU/mL) BDD-FVIII, without any requirement for antigen presentation. *In vivo*, naïve BALB/c *F8*e16$^{-/-}$ recipient mice were infused with 5x10^{5} sorted TRuC Tregs or polyclonal thymic derived (t)Tregs (5x10^{5} or 1x10^{6}) followed by 8 weekly IV injections of 1.5 IU BDDFVIII. FVIII TRuC Tregs were more effective at suppressing inhibitor formation as compared to polyclonal tTregs. 7 out of 8 animals in the TRuC Treg group did not develop detectable inhibitors (0.23±0.23 BU/ mL) at 4 weeks compared 8 out of 8 in the control group (69.42±33.99 BU/mL). At 8 weeks, control mice had a mean inhibitor titer of 151.4±48.6 BU/mL compared to 15.4±10.4 BU/mL in the TRuC Treg group. αFVIII IgG1 titers were also significantly lower in the TRuC Treg group (5238±3862 ng/mL) compared to the 5x10^{5} (28429±3862 ng/mL, p=0.0042) or 1x10^{6} (21821±8020 ng/mL, p=0.0438) tTreg groups at 8 weeks, suggesting a more sustained tolerogenic effect. In conclusion, this study suggests that adoptive cellular therapy with antigen specific engineered TRuC Tregs is able to suppress antibody formation against the soluble therapeutic protein FVIII in an MHC-independent manner, in spite of persisting only transiently in vivo (~7 days). More studies are required on regulating activation thresholds to maintain optimal suppressive function in engineered Tregs.

140. Functional Benefit of Mitochondrially Augmented HSPCs: Improved Engraftment and Alterations in Immune Cell Differentiation

Noa Sher[1], Elad Jacoby[2,3], Moriya Ben Yakir-Blumkin[1], Shiri Blumenfeld-Kan[1], Yehuda Brody[1], Amilia Meir[2], Ayelet Shabtay-Orbach[1], Gat Pozner[1], Tina Napso[1], Natalie Yivgi-Ohana[1], Amos Toren[2,3]

[1]Minovia Therapeutics Ltd, Tirat Hakarmel, Israel,[2]Pediatric Hematology and Oncology, Cell Therapy Center, The Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel,[3]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

mtDNA associated disorders (either deletions, mutations or depletion) lead to multisystemic disease, often severe at a young age, with no disease-modifying therapies. In mitochondrial augmentation therapy (MAT), hematopoietic stem and progenitor cells (HSPCs) are enriched with healthy donor mitochondria ex vivo. This process allows cells to import mitochondria harboring full length mtDNA, resulting in increased mitochondrial content and improved function. HSPCs were chosen as recipient cells for MAT due to their demonstrated ability to alleviate systemic (hematological and non-hematological) pathologies. We demonstrated that mitochondrial augmentation of HSPCs is dose-dependent and confers functional benefit in both healthy donor and patient-derived HSPCs. We showed that HSPCs can be enriched with up to 34.9% exogenous mtDNA, resulting in increased oxygen consumption rate. To assess the potential effects of mitochondrial augmentation on human HSPCs, we used a humanized NSGS mouse transplanted with cord-blood derived CD34+ cells from a patient with Pearson Syndrome (PS), a mtDNA deletion syndrome. In this non-conditioned model, mitochondrially augmented CD34+ cells had improved long-term engraftment in the NSGS mice, as confirmed by flow cytometry and dPCR. Multi-lineage hematopoietic potential of engrafted cells was demonstrated, and animals which received mitochondrially enriched cells had significantly higher percentages of human CD3+ T cells. To investigate persistence in an immunocompetent animal, we developed a mouse model in which all nuclei were labelled with red fluorescence (Tomato) and all mitochondria were labelled with green fluorescence (Dendra). Recipient Polg mice had a point mutation in the mitochondrial DNA polymerase gene, leading to accumulation of mtDNA mutations. We were able to show long term persistence of daughter cells without

preconditioning treatment. Continuous in vivo transfer of exogenous mitochondria to recipient host hematopoietic cells was observed and persisted up to 4.5 months post transplantation, the last time point tested. Cells of both myeloid and lymphoid lienage were recipients of mitochondria in the peripheral blood. Taken together, these data provide evidence supporting feasibility of augmentation of human HPSCs and for the potential of MAT as a therapeutic modality for the treatment of mitochondrial disorders.

141. Memory Enriched Epstein-Barr Virus {EBV} Specific T-Cells with Broader Target Antigen Repertoire for the Treatment of EBV+ Malignancies

Sandhya Sharma[1,2], Naren Mehta[1], Kathan Parikh[1], Ayea
El-Ghazali[1], Mae Woods[1], Tim Sauer[1], Huimin Zhang[1], Birju Mehta[1], Vicky Torrano[1], Bambi Grilley[1], Helen Heslop[1,3,4], Cliona Rooney[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Graduate School of Biomedical Sciences, Baylor College of Medicine, Houston, TX,[3]Houston Methodist, Houston, TX,[4]Texas Childrens Hospital, Houston, TX

Almost 40 % Hodgkin's and Non-Hodgkin Lymphoma {HL/NHL} patients carry the EBV genome in a type 2 latency state in which 4 viral type-2 latency antigens {T2-Ag} are expressed. T2-Ag-specific T-cells from lymphoma patients are difficult to expand, likely because they are rendered anergic in the tumor microenvironment, and circulate with low frequency. In a clinical trial-NCT01555892 using patient-derived, EBV-specific T cells {EBVSTs} to target these tumors, ~25% of EBVST lines failed our manufacturing criteria due to lack of antigen specificity or failure to grow. Even in successfully expanded lines, over 50% recognized only 1 or 2 antigens, which could lead to tumor escape by antigen modulation. We ***hypothesized*** that low antigen specificity could result from the expansion of non-specific bystander cells in our cultures, and that enrichment of memory T-cells prior to culture as well as stimulation with additional viral tumor antigens could improve their potency. To enrich for memory T-cells, we **depleted the CD45RA+ fraction** of PBMCs that contains naïve T-cells, T-regs and NK-cells prior to EBVSTs generation. To broaden the tumor-specific T-cell repertoire, we evaluated EBV lytic cycle antigen expression in HL biopsies and combined EBV lytic antigen and T2-Ag peptide libraries to generate **broad repertoire {BR}-EBVSTs** with specificity to both in γ-IFN ELIspot assays. Depletion of CD45RA+ cells prior to EBVSTs generation increased the frequency of T2-Ag specific T-cells by 2-10 fold, and enhanced proliferation and cytotoxicity compared to EBVSTs generated from unfractionated PBMCs. Most importantly, we restored the ability of patient EBVSTs to respond to EBV T2-Ags. This unexpected finding suggested that CD45RA+ cells are capable of inhibiting the reactivation and expansion of T2-Ag specific T-cells. In support of our proposal to target both T2- and lytic antigens, we identified both T2-latent and lytic cycle transcripts in HL biopsy samples and demonstrated that BR-EBVSTs could be generated by stimulation with a combination of T2- and lytic-cycle antigens. Following adoptive transfer into an EBV+ murine xenograft model, autologous BR-EBVSTs cleared tumor more rapidly {average by

day 15} than T2-EBVSTs {average by day 30} and better limited metastatic spread. Notably human GM-CSF and IFN-γ serum levels were 2-fold or higher in mice treated with BR-EBVSTs, which should create a more pro-inflammatory tumor milieu leading to increased tumor killing and epitope spreading in lymphoma patients. **BR-EBVSTs from memory enriched CD45RA+ subset depleted PBMCs** are now under evaluation in our amended clinical trial. We generated EBVSTs with high antigen specificity from 7/7 lymphoma patients and treated five, including the first patient whose EBVSTs had failed manufacturing using the previous protocol {Fig 1.}

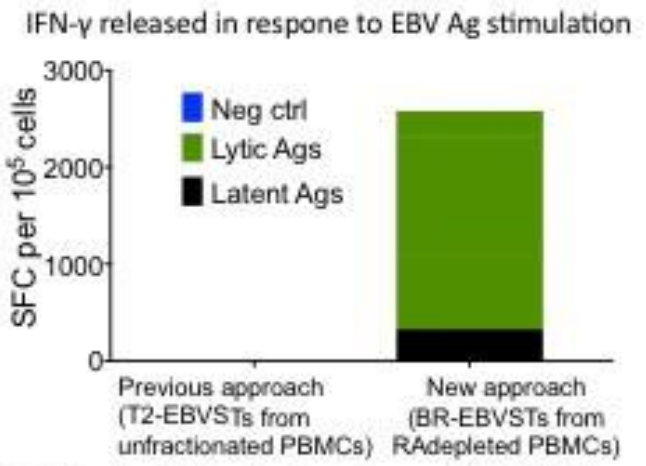


Fig 1.) Bar Graph illustrating the antigen specificity of EBVST lines generated for patient 1 by two different approaches. Y-axis represents the Spot Forming Cells (SFC) as a measure of number of cells that secreted IFN-γ in response to EBV antigen stimulation.

We have observed an increase in the frequency of T-cells recognizing both T2- and lytic antigens in patient PBMCs post-infusion for up to 3 months. Long-term follow-up and comparison with patients who received T2-EBVSTs from unfractionated PBMCs will determine if these changes indeed produced a more potent clinical product.

142. Non-Viral Engineering of CAR-NK Cells Using the *TC Buster* Transposon System™

Emily J. Pomeroy, Walker S. Lahr, Beau R. Webber, Branden S. Moriarity

Pediatrics, University of Minnesota, Minneapolis, MN

Immunotherapy with T cells and NK cells modified with viral vectors to express a chimeric antigen receptor (CAR) has shown remarkable efficacy in clinical trials. However, viral vectors are limited in their cargo size, carry the risk of insertional mutagenesis, and large-scale manufacturing for clinical use can be cost-prohibitive. Thus, CAR delivery via DNA transposon engineering has been pursued as an alternative due to convenient and cost-effective production and a better safety profile. Engineering via transposition is accomplished using a two-component system: a plasmid containing a gene expression cassette flanked by inverted terminal repeats (ITRs) and a transposase enzyme that binds to the ITRs and integrates the transposon into the genome. Here, we sought to use the newly developed *TC Buster*™ (Bio-Techne) transposon system to deliver a transposon containing a CD19-CARDHFR-EGFP expression cassette (3.7 kb transposon, figure 1A) to primary human peripheral blood (PB) NK cells. However, the use of transposons in NK cells has been very limited due to DNA toxicity and induction of a type I interferon response. Thus, we optimized methods to avoid this, including using DNase in recovery media and delivery via Nanoplasmid vectors which have a small (<500 bp) backbone, high supercoiling, and are regulatory compliant. We optimized activation, electroporation, recovery, and expansion

conditions to achieve 49.6% (±4.64%) integration efficiency using an evolved hyperactive *TC Buster*™ transposase (Hyp-TCB) (Figure 1B). Our cargo also contained a mutant dihydrofolate reductase (DHFR) which allowed us to select for stable transposon integration using methotrexate (MTX). Our optimized protocol achieves manufacturing in 20 days and results in 99.2% (±0.5%) CAR+ NK cells expanded 837.9-fold (±88.6) from input (Figure 1C, 1D). We tested CAR-NK cells in functional assays against CD19-expressing Raji cell lines. CAR-NK cells produced significantly more IFNγ and TNFα than CAR-negative NK cells when co-cultured with Raji (Figure 2A, 2B). CAR-NK cells also expressed significantly more CD107a on their surface, a marker of degranulation (Figure 2C). In killing assays, CD19-CAR-NK cells killed over 90% of Raji cells in 24 hours at effector-to-target ratios as low as 1:3 (Figure 2D). Our work provides a platform for robust delivery of multicistronic, large cargo via transposition to primary human PB NK cells. We are currently using this platform to deliver larger cargo of interest that far exceeds the carrying capacity of viral vectors. We demonstrate that CAR-NK cells can be enriched using MTX selection, while maintaining high viability and function, and they can be expanded to clinically relevant numbers in a matter of weeks. In recent experiments we have demonstrated successful delivery of *TC Buster*™ transposons for large cargo integration with CRISPR/Cas9 for targeted gene knockout in a single electroporation event. This non-viral approach for multiplex editing of NK cells represents a versatile, safer, and more cost-effective option for the manufacture of CAR-NK cells compared to viral delivery.

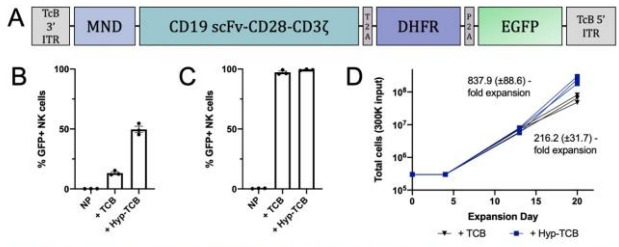


Figure 1. Delivery of a CD19-CAR-DHFR-GFP transposon using the evolved hyperactive TC Buster Transposon System. (A) The 3.7 kb transposon flanked by TC Buster ITRs, containing an MND promoter, CD19 CAR, mutant DHFR selection gene, and enhanced GFP. Elements are separated by 2A self-cleaving peptides. Transposon was delivered in a nanoplasmid (NP) backbone. (B) Primary human peripheral blood NK cells (n=3 human donors) were electroporated with the nanoplasmid (NP) alone or in combination with mRNA encoding either TC Buster (TCB) or the evolved hyperactive mutant TC Buster (Hyp-TCB). Two days after electroporation, NK cells were expanded with mbIL21-expressing feeder cells for 1 week. After expansion, GFP expression was measured by flow cytometry. (C) NK cells were expanded again with mbIL21-expressing feeder cells for one week in media containing 250 nM methotrexate (MTX) and then GFP expression was measured by flow cytometry. (D) Fold expansion was calculated over the course of the experiment.

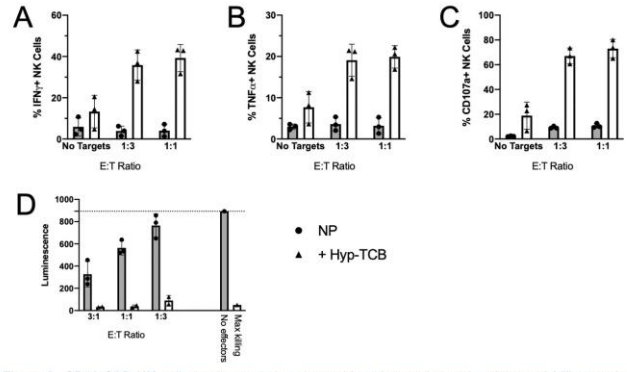


Figure 2. CD19-CAR-NK cells engineered via transposition show enhanced activity and killing against CD19+ target cells. MTX-selected NK cells (n=3 human donors) expressing CD19-CAR were co-cultured with luciferase-expressing, CD19-positive Raji target cells. (A-C) After co-culture, NK cells were analyzed by flow cytometry for expression of IFNγ, TNFα, and CD107a. (D) Target cell killing was measured by luciferase expression after co-culture.

143. Adoptively Transferred, *In Vitro* Generated Alloantigen-Specific Type 1 Regulatory T (Tr1) Cells Persist Long-Term *In Vivo*

Alma-Martina Cepika[1], Pauline P. Chen[1], Rajni Agarwal[1], Gopin Saini[1], David M. Louis[2], Laura C. Amaya-Hernandez[3], Liwen Xu[4], Parveen Shiraz[4], Keri M. Tate[5], Dana Margittai[5], Neehar Bhatia[5,6], Everett Meyer[4], Alice Bertaina[1], Mark M. Davis[2,7,8], Rosa Bacchetta[1,6], Maria Grazia Roncarolo[1,3,6]

[1]Pediatrics, Stanford University School of Medicine, Stanford, CA,[2]Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine, Stanford, CA,[3]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA,[4]Medicine, Stanford University School of Medicine, Stanford, CA,[5]Laboratory for Cell and Gene Medicine, Stanford University School of Medicine, Stanford, CA,[6]Center for Definitive and Curative Medicine, Stanford University School of Medicine, Stanford, CA,[7]Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA,[8]Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Allogeneic, HLA-mismatched hematopoietic stem cell transplantation (allo-HSCT) can be a life-saving treatment for many high-risk hematological malignancies, as well as for certain non-malignant disorders. However, the largest obstacle to wide-spread use of allo-HSCT is its frequent and life-threatening side-effect, graft-vs-host disease (GvHD). GvHD is caused by HSCT donor-derived effector T (Teff) cells that attack patient's HLA-mismatched healthy tissues. GvHD can be treated, but all standard-of-care treatments also induce generalized immunosuppression, increasing the risk of relapse, infection, and death. To alleviate GvHD without causing generalized immunosuppression, we aim to leverage type 1 regulatory T (Tr1) cells. Tr1 cells are inducible, IL-10+FOXP3- subset of regulatory T cells that was shown to correlate with tolerance in allo-HSCT patients, and prevent GvHD in mice. Tr1 cells suppress the activity of Teff cells, which cause GvHD *in vivo*, through soluble IL-10 and co-inhibitory surface receptors. We have generated alloantigen-specific Tr1 cells by co-culturing HSC donor-derived CD4 T cells with patient-derived tolerogenic dendritic cells, which present patient alloantigens and induce

differentiation of CD4 T cells into Tr1 cells. During the differentiation, Tr1 cells clonally expand, developing a highly restricted TCR repertoire. The final Tr1-enriched cell product, called T-allo10, is anergic and suppresses Teff cell proliferation specifically in response to alloantigens, but not irrelevant antigens (Cepika AM, Chen PP et al, J ImmunoTher Cancer 2020; Vol. 8, S3:146). T-allo10 cells are being tested for safety in a phase I clinical trial in children and young adults with hematological malignancies undergoing allo-HSCT (ClinicalTrial.gov ID: NCT03198234). In this trial, T-allo10 cells are adoptively transferred one day before allo-HSCT, and patients are immunomonitored in 19 time points from day 0 to 360. One year-post treatment, the first three patients

are alive, have met the safety criteria, and are GvHD and cancer-free (Agarwal R et al, Biol Blood Marrow Transplant 2020; Vol. 26 (3): S272-S273). To demonstrate the long-term survival of adoptively transferred Tr1 cells in these first three patients, we FACS-sorted the highly purified Tr1 cells from patient's T-allo10 cell product, and compared their TCR repertoire to non-Tr1 cells from the same product, parental CD4 T cells, and control Teff cells. We determined the top 20 most highly enriched TCR clonotypes of Tr1 cells, and then compared this data to the patient whole blood TCR repertoire in all 19 time-points. Using this method as complementary to peripheral Tr1 cell detection by flow cytometry, we demonstrate that Tr1 cells persist in patients for up to 1-year post adoptive transfer. This implies that Tr1 cells represent a durable cell therapy option to modulate antigen-specific tolerance.

144. Endothelial Progenitor Cells Engineered to Overexpress Endothelial NO-Synthase May Improve Infarct Healing: Results from the Enhanced Angiogenic Cell Therapy - Acute Myocardial Infarction (ENACT-AMI) Trial

Duncan Stewart[1], Michael Kutryk[2], Hung Q. Ly[3], Christopher A. Glover[4], Alexander Dick[5], Kim Connelly[2], Shaun G. Goodman[2], Howard Leong-Poi[2], Leslie Carlin[1], Rose Gaudet[1], Monica Taljaard[6], David Courtman[1]

[1]Sinclair Centre for Regenerative Medicine, Ottawa Hospital Research Institute, Ottawa, ON, Canada,[2]St. Michael's Hospital, Toronto, ON, Canada,[3]Cardiology, Montreal Heart Institute, Montreal, QC, Canada,[4]Cardiology, University of Ottawa Heart Institute, Ottawa, ON, Canada,[5]University of Ottawa Heart Institute,
Ottawa, ON, Canada,[6]Ottawa Methods Centre, Ottawa Hospital Research Institute, Ottawa, ON, Canada

Introduction: Despite modern reperfusion therapies for acute myocardial infarction (AMI), many patients are left with a significant area of injury leading to scar formation that contributes to a dysfunctional myocardium and the development of heart failure. The goal of the Enhanced Angiogenic Cell Therapy-AMI (ENACTAMI) trial was to determine whether intracoronary delivery of early outgrowth endothelial progenitor cells (EPCs) engineered to overexpress endothelial NO-synthase (eNOS) would improve global left ventricular ejection fraction (LVEF) (primary outcome) and infarct size as assessed by cardiac MRI (CMR) in patients with LAD territory ST elevation myocardial infarction treated with evidence-based therapies. **Methods**: ENACT-AMI (NCT00936819) was a double-blind placebocontrolled trial in which participants were randomized to one of 3 arms:

1) saline placebo; 2) EPCs; or 3) eNOS-transfected EPCs. EPCs were derived from circulating mononuclear cells by 7-8 days of differential culture on fibronectin in EGM supplemented with endothelial growth factors using fully sourced GMP-compatible reagents. Transfection was performed using a plasmid DNA vector (pVax) containing the human eNOS sequence combined with JetPEI (Polyplus). Target sample size was 100 participants. Groups were compared using analysis of covariance (ANCOVA). **Results**: The trial was terminated due to slow recruitment after 47 patients were enrolled at three Canadian sites over six years (n=18 placebo, n=15 EPCs; n=14 eNOS-transfected EPCs). The groups were comparable with respect to demographic variables including age (56.1±9.7 years), cardiac risk factors, pre-existing cardiac disease, peak CK and troponin values and baseline LVEF by cardiac magnetic resonance imaging (40.7±9.3). Intracoronary cell delivery (20M cells; 20±5 days post-AMI) was well tolerated. At 6 months, there were no significant differences in the primary endpoint of LVEF between groups (mean difference between the average of the two EPC groups vs. placebo: 0.5% [95% Confidence Interval (CI) 2.9% to 3.9%). The secondary outcome of left ventricular infarct mass indexed to LV size at 6 months demonstrated no significant difference between the average of the two EPC groups versus placebo (p=0.72); however, a significant difference was seen in those receiving eNOS-transfected EPCs compared to EPCs (-6.6; CI -12.0 to -1.1, p=0.02). Only four major cardiovascular events were observed over an average follow up of 4.1±1.6 years, and these were equally distributed across groups. **Conclusions**: While there were no significant differences in LVEF, the results of the ENACT-AMI trial suggest that intracoronary delivery of gene-enhanced EPCs reduced infarct size and LV diastolic diameter in patients with large anterior wall AMI consistent with improved scar healing. These findings have important implications for remodelling and require confirmation in larger clinical trials.

CAR-Based Cancer Gene Therapy

145. Chimeric Antigen Receptor Macrophages (CAR-M) Induce Anti-Tumor Immunity and Synergize with Immune Checkpoint Inhibitors in Pre-Clinical Solid Tumor Models

Stefano Pierini[1], Rashid Gabbasov[1], Alison Worth[1], Linara Gabitova[1], Daniel Blumenthal[1], Yumi Ohtani[1], Olga Shestova[2], Maksim Shestov[2], Saar Gill[2], Sascha Abramson[1], Thomas Condamine[1], Michael Klichinsky[1]

[1]Carisma Therapeutics, Philadelphia, PA,[2]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA

Despite the remarkable efficacy achieved by CAR-T cell therapy in hematologic malignancies, translating these results for solid tumors remains challenging. We previously developed human CAR-M and demonstrated that adoptive cell transfer of CAR-M into xenograft models of human cancer controls tumor progression and improves overall survival[1]. Given that CAR-M are professional antigen presenting cells, we established a fully immunocompetent syngeneic mouse model to evaluate the interaction of CAR-M with the tumor microenvironment (TME) and the potential for induction of a systemic anti-tumor immune response. Murine bone marrowderived CAR-expressing macrophages (muCAR-M) were efficiently engineered to express an anti-huHER2 CAR using the chimeric adenoviral vector Ad5f35. In addition to efficient gene delivery,

Ad5f35 transduction promoted a pro-inflammatory (M1) phenotype in murine macrophages. MuCAR-M, but not control untransduced (UTD) macrophages, specifically phagocytosed HER2+ target cancer cell lines and killed HER2-expressing murine CT26 colorectal and human AU-565 breast cancer cells in a dose-dependent manner. Moreover, CAR-M induced MHC-I and MHC-II expression on tumor cells and cross-presented tumor-associated antigens (TAA) resulting in CD8+ T cell activation. To evaluate muCAR-M in an immunocompetent *in vivo* setting, BALB/c mice were engrafted with subcutaneous CT26HER2+ tumors and treated with HER2-CAR or UTD macrophages. CAR-M treated mice showed significant tumor control and improved survival compared to control groups. Analysis of the TME showed increased intratumoral immune infiltration as well as an increase in T cells reactive to the CT26 MHC-I antigen gp70, indicating enhanced epitope spreading. Mice that achieved complete responses (CRs) after CAR-M therapy were protected against antigen-negative relapse in a HER2- CT26 (CT26-Wt) rechallenge model. This anti-tumor immune response was CD3+ T cell-mediated and suggested induction of long-term memory against TAA. To evaluate the systemic anti-tumor immune response, we simultaneously engrafted BALB/c mice with CT26-HER2+ and CT26-Wt tumors on opposite flanks and treated mice with local administration of CAR-M to the HER2+ tumors. After CAR-M treatment, 75% of mice cleared their CT26-HER2+ tumors and the growth rate of the contralateral CT26-Wt tumors was significantly reduced, demonstrating an abscopal effect. Given the impact of CAR-M on the endogenous adaptive immune system, we evaluated the combination of CAR-M with PD1 checkpoint inhibitor therapy in the CT26-HER2 model, which is resistant to anti-PD1 monotherapy, and found that the combination further improved tumor control and overall survival. These results demonstrate that CAR-M reprogram the TME, induce epitope spreading, and orchestrate a systemic immune response against solid tumors. Moreover, our findings provide rationale for the combination of CAR-M with immune checkpoint inhibitors. The antiHER2 CAR-M, CT-0508, is under evaluation in a phase I clinical trial for patients with HER2 overexpressing solid tumors (NCT04660929).

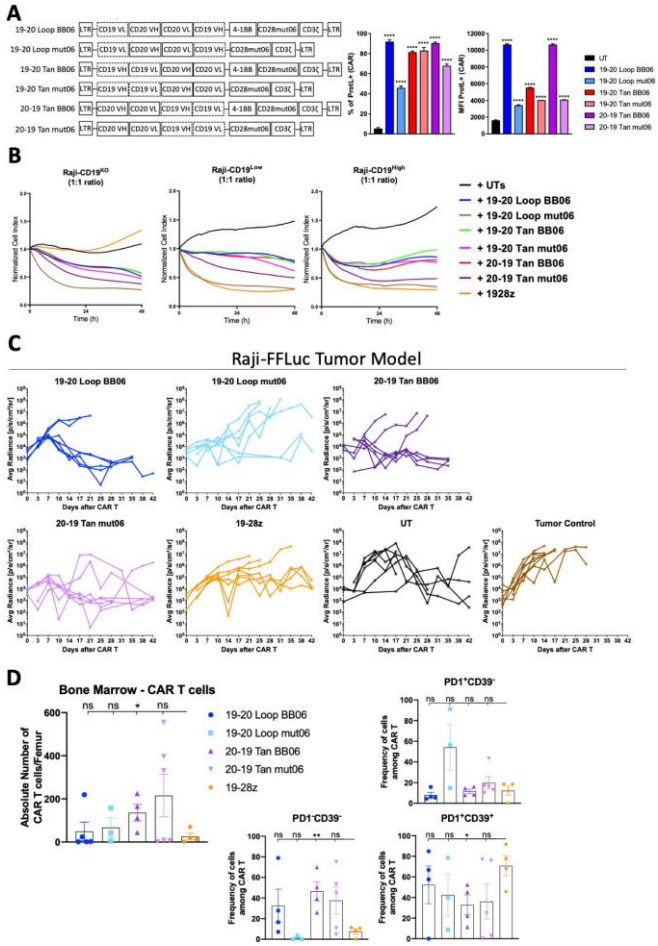
1. Klichinsky M, Ruella M, Shestova O, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. Nat Biotechnol. 2020;38(8):947-953.

146. Bivalent CD19/CD20-Specific CAR T Cells with 4-1BB and Mutated CD28 Co-Stimulatory Domains Show Enhanced Function

Emiliano Roselli, Gongbo Li, Kristen Spitler, Justin Boucher, Kayla M. Reid, Sae Bom Lee, Nhan Tu, Marco L. Davila

Immunology, Moffitt Cancer Center, Tampa, FL

Despite the high response rates of CD19 CAR T cell therapy, the loss or downregulation of CD19 has been reported as a common mechanism of tumor resistance. Current strategies focused on overcoming antigen escape include the use of CAR T cells able to recognize more than one target antigen. Additionally, the choice of co-stimulatory domains impact CAR T cell expansion, persistence and antitumor function. Our recent study found that mutating the CD28 endodomain to retain signaling only through the PYAP motif (mut06) rendered enhanced antitumor activity to CAR T cells, which was in part due to resistance to T cell exhaustion (Boucher et al. 2020). For this work we developed bi-specific constructs encoding for single bivalent CAR molecules able to recognize both human CD19 (FMC63) and CD20 (Leu16) in a loop or tandem configuration. To take advantage of the bi-specificity and the enhanced co-stimulatory domain we developed second generation bivalent CARs with mutated CD28 (mut06) and third generation CARs containing both 4-1BB and mut06 (BB06). We determined efficient surface CAR expression in all constructs and increased central memory phenotype in third generation BB06 CARs (Figure 1A). We detected secretion of significant levels of IFNγ, IL-2, TNFα, and minimal levels of IL-6 upon engagement to either CD19 or CD20 with the highest levels observed when both antigens were present. We determined in vitro cytotoxicity by a real-time assay (RTCA) against either antigen alone or both and observed significant lytic effect of all constructs with second generations (mut06) CARs showing a faster response (Figure 1B). We then analyzed in vivo antitumor function of these bivalent CAR T cells against a xenograft model of Raji-FFLuc. Third generation CAR T cells with mutated CD28 (BB06) showed a more effective tumor growth control compared to mono-specific anti-CD19 with unmutated CD28 co-stimulation (1928z)(Figure 1C). Importantly, these BB06 CAR T cells showed increased persistence in the mice at day 45 post-inoculation compared to 1928z. Furthermore, BB06 CAR T cells displayed significantly lower frequency of cells expressing the exhaustion-associated markers PD1, TIM3 and CD39 compared to 1928z and second-generation mut06 cells (Figure 1D). In a subsequent in vivo experiment using Raji cells only expressing CD20 (Raji-CD19KO) we were able to validate our previous results showing 20-19 tandem BB06 as the most effective construct against this CD19KO model. To date our results demonstrate that these bi-specific CAR T cells are able to recognize and trigger an effector function upon engagement of CD19 and/or CD20 and, most importantly that the combination of 4-1BB and this new mutated form of CD28 as co-stimulation enhance the antitumor function of cells. This work supports the concept that CAR T cell therapies can be improved by optimizing their design, including this dual antigen-binding domain and modified co-stimulatory motifs.



147. Combining IAP Inhibitors with CAR T Cell Therapy to Treat Glioblastoma

Edward Z. Song[1], Benjamin I. Philipson[1], Radhika Thokala[1,2], Zev A. Binder[2], Donald M. O'Rourke[2], Michael C. Milone[1]

[1]Department of Pathology and Laboratory Medicine and Center for Cellular Immunotherapies, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Antigen heterogeneity is one of the major obstacles to successful chimeric antigen receptor (CAR) T cell treatment in solid tumors including glioblastoma, as antigen-negative tumor cells could escape from the CAR-T cells. To address this issue and improve the efficacy of CAR-T cell therapy for glioblastoma, we are developing an approach that combines IAP inhibitors, a new class of small molecules, with CAR-T cells to treat glioblastoma. IAP inhibitors can induce the degradation of inhibitor of apoptosis (IAP) proteins. By antagonizing IAP proteins, IAP inhibitors could sensitize glioblastoma cells, including antigen-negative cells, to apoptosis induced by CAR-T cell-derived tumor necrosis factor (TNF). Meanwhile, IAP inhibitors may promote CAR-T cell survival, proliferation and cytokine production including TNF via NF-kB inducing kinase (NIK)-mediated NF-κB pathways. We demonstrate that the IAP inhibitor Birinapant, currently in early phase clinical studies, induces the degradation of cellular IAP1 (c-IAP1) in the human glioblastoma cell line M059K, and exogenous TNF and Birinapant synergistically kill M059K via apoptosis. We further show that while CAR-T cells targeting glioblastoma antigen epidermal growth factor receptor variant III (EGFRvIII) do not directly kill M059K parental cells lacking EGFRvIII expression, Birinapant enhances the "by-stander" cell death of M059K parental cells when co-cultured with M059K cells transduced with EGFRvIII and the anti-EGFRvIII CAR-T cells, which express TNF when stimulated. Furthermore, our data show that conditioned medium from activated CAR-T cells mediates M059K cell death in the presence of Birinapant, and this death is partially blocked by TNF neutralization by infliximab, supporting the role of TNF in the mechanism of by-stander killing. In addition, we investigated how the IAP inhibitor Birinapant affects CAR-T cell functions. Exploiting an *ex vivo* repetitive stimulation assay, we show that Birinipant promotes CAR-T cell proliferation as measured by a higher fraction of cells in S-phase and also enhances TNF production by the CAR-T cells with prolonged antigen stimulation. These effects are associated with the activation of both the canonical and non-canonical NF-kB pathways. Glioblastoma xenograft models with heterogeneous tumor antigen expression are currently being developed to evaluate the efficacy of combining CAR-T cells with Birinipant *in vivo* in order to prevent escape due to antigen heterogeneity. In conclusion, our results support the potential for combining IAP inhibitors such as Birinipant as a novel approach to addressing antigen heterogeneity and tumor escape that represent a major hurdle to CAR-T cell-based immunotherapies.

148. B-CLL-Mediated Insufficient Activation Is CAR-Independent

McKensie A. Collins, Weimin Kong, In-Young Jung, Stefan Lundh, Joseph A. Fraietta, J. Joseph Melenhorst

University of Pennsylvania, Philadelphia, PA

Background: Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the western world, accounting for nearly 1/3rd of adult leukemia diagnoses. While standard-of-care chemoimmunotherapies are initially successful in treating this disease, the lack of curative therapeutic options means that most patients will ultimately succumb to their disease. Chimeric Antigen Receptor (CAR) T cell therapy has proven effective, but only in a small subset of patients. Improving response rates to this therapy will provide muchneeded curative options for this patient population. **Findings:** We have previously shown that the inability of CLL cells to activate CAR T cells drives this lack of response. In a serial re-stimulation model, we observed defects in both CD19- and ROR1-directed CAR T cell proliferation, cytokine production, and cytotoxicity. Flow cytometry showed that CLL-stimulated CAR T cells maintained an un-activated profile, suggesting that CLL cells fail to stimulate CAR T cells rather than rendering them non-functional. We further showed that these defects were non-permanent and could be rescued by stimulating CAR T cells with an artificial antigen presenting cell (aAPC) with or without the presence of CLL cells. Immunophenotyping of our B-CLL biobank showed that the majority of CLL cells (18/20 patients, 90%) express the IL-

2 receptor alpha chain (CD25) at a high level. We hypothesized that CD25 may be part of an active IL-2R signaling complex, allowing CLL cells to outcompete the CAR T cells for IL-2, limiting their activation and explaining the observed phenotype. We supplemented IL-2 into CLL/CAR T cell co-cultures which rescued the proliferative capacity of the CAR T cells and partially rescued cytokine production. Further, we performed intracellular cytokine staining of CAR T cells stimulated with aAPCs and CLL cells. Interestingly, we found that CLL cells fail to stimulate IL-2 production after either a 6- or 12-hour incubation, disproving the IL-2 sinking hypothesis. However, we did observe low-level CD107a acquisition, suggesting that the CAR T cells retain some cytotoxic function. We next proposed that low-level expression of co-stimulatory or adhesion molecules on CLL cells may impair CAR T cell activation. Immunophenotyping of the CLL cells showed low level expression of molecules such as CD54, CD80, and CD86. We hypothesized that up-regulation of these molecules could improve CAR T cell targeting of CLL cells. We activated CLL cells via CD40L and IL-4 which resulted in subsequent up-regulation of CD54, CD58, CD80, and CD86 as well as other molecules. Stimulation of CAR T cells with these activated CLLs enhanced CAR T cell proliferation and cell-conjugate formation, indicating stronger cell-to-cell interactions. Therefore, improving CLL stimulatory capacity can rescue T cell dysfunction. To assess whether IL-2 addition and CD40 ligation were synergistic, we combined the two assays; however, we saw no additional improvement over IL-2 addition alone. We showed that rescue via either IL-2 addition or CD40 ligation was not CAR-specific, as we observed similar defects and rescue with both a ROR1-targeting CAR and the gold standard CD19-targeting CAR. **Conclusions:**These data indicate that CAR T cell dysfunction in CLL is mediated by insufficient activation of CAR T cells rather than true defects in cell function. Improving the stimulatory capacity of CLL cells via IL-2 addition or CD40 activation may enable better clinical responses. Additionally, the requirement for enhanced co-stimulation and adhesion even in a second-generation CAR T cell containing a co-stimulatory signaling domain is a unique finding. Further, the conserved effect between CD19- and ROR1-targeting CARs suggests that these results may be broadly applicable to CAR T cell therapies, and may be relevant in other indications beyond CLL.

149. CAR Design and Expression Determine Hyper-Proliferative States in TET2 Deficient T Cells

Nayan Jain[1], Zeguo Zhao[1], Archana Iyer[1], Michael Lopez[1], Judith Feucht[2], Richard Koche[1], Julie Yang[1], Yingqian Zhan[1], Michel Sadelain[1]

[1] Memorial Sloan Kettering Cancer Center, New York, NY,[2]University of Tübingen, Tübingen, Germany

TET2 disruption through a chance integration of a 4-1BB chimeric antigen receptor (CAR) lentiviral vector led to the emergence of a dominant CAR T cell clone, which coincided with tumor clearance in a chronic lymphocytic leukemia (CLL) patient (Fraietta et. al. Nature 2018). The enhanced proliferative ability ascribed to the *TET2* disruption opened the possibility of treating patients with lower doses of TET2 deficient CAR T cells than are currently required. In a pre-clinical murine model of human acute lymphoblastic leukemia (ALL), we studied the effect of CAR design in determining the effect of *TET2* editing on T cell phenotype and anti-tumor efficacy. To assess the effect of *TET2* disruption on CAR T cell therapeutic efficacy, we treated immune deficient mice bearing the human ALL cell line, NALM6, with limiting doses of either *TET2* edited (CRISPR/Cas9) or unedited CAR T cells. We investigated the two most widely used second generation CD19-specific CARs encompassing the costimulatory domain of either CD28 (Rv-1928z) or 4-1BB (Rv-19BBz). *TET2* editing enhanced the anti-tumor efficacy of Rv-19BBz CAR T cells and promoted the acquisition of a central memory phenotype. In contrast, *TET2* editing did not alter the anti-tumor efficacy of Rv-1928z CAR T cells or their differentiation state. As Rv-1928z CAR T cells have a stronger induction of effector differentiation than Rv-19BBz CAR T cells, this divergence led us to hypothesize that *TET2* editing acts in concert with CAR signaling to remodel the T cell phenotype. To test this hypothesis, we edited *TET2* in two additional CAR designs that have been shown to limit T cell effector differentiation over retrovirally encoded CD28 costimulated CARs: 1928z driven by the *TRAC* promoter (*TRAC*-1928z) and Rv-1928z co-expressing the 4-1BB ligand (Rv-1928z-41BBL). Indeed, disruption of *TET2* enhanced the anti-tumor efficacy of both these CAR T cells and increased their early central memory phenotype. However, *TET2* edited CAR T cells, over time, attain a hyper-proliferative phenotype with a near total loss of effector function. This state was consistently associated with biallelic *TET2* editing. The frequency with which *TET2* edited T cells achieved hyper-proliferation depended on the signaling properties of the CAR receptor. This hyper-proliferative state is associated with sustained upregulation of cell cycle factors and shares features with some T cell leukemia/lymphoma. Exome analysis identified point mutations and chromosomal aberrations in hyper-proliferative cells, but they were not conserved across different populations, suggesting an alternate mechanism as a likely candidate for driving the hyper-proliferation. Chromatin accessibility analysis revealed that loss of *TET2* sets an epigenetic state that allows for sustained elevated levels of BATF3, which in turn drives a MYC-dependent proliferative program in *TET2* deficient CAR T cells. Our study shows that disruption of *TET2* may enhance the proliferation and persistence of T cells, depending on their CAR receptor, but that *TET2* deficient CAR T cells eventually uncouple their proliferative program from effector function thus impairing their therapeutic potency.

150. Tumor-Responsive, Multifunctional CARNK Cells Cooperate with Impaired Autophagy to Infiltrate and Target Glioblastoma

Jiao Wang[2], Sandro Matosevic[1,2]

[2] Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN,[2]Center for Cancer Research, Purdue University, West Lafayette, IN

Background: Tumor antigen heterogeneity, a severely immunosuppressive tumor microenvironment (TME), and

suppression of NK cell function: tumor-associated proteases (TAP) mediated site-specific release of anti-CD73 scFv which can inhibit the activity of CD73 independently of CAR signaling and decrease the local concentration of adenosine (**Fig.1A-C**). To address insufficient homing of NK cells into the tumor bed, we combined these cells with chloroquine (CQ) as an adjuvant to inhibit autophagy in GBM and evaluated these cells against patient-derived intracranial GBM models in vivo (**Fig.1D**). **Results:** When administered with CQ, CD73.mCARpNK cells could effectively target orthotopic patient-derived GBM xenografts, demonstrating effective anti-tumor responses (**Fig.1E** and **F**), potent inhibition of CD73 expression (**Fig.1G** and **H**) and remarkable suppression of adenosine production in the local tumor (**Fig.1I** and **J**). In addition, we also unveiled a complex reorganization of the immunological profile of GBM induced by inhibiting autophagy. In particular, pharmacologic impairment of the autophagic process promoted a significant secretion of chemokines, including CCL5 and CXCL10, which were favorable to NK cell infiltration (**Fig.1K**). In response to combination therapy with CD73.mCAR-pNK cells and CQ, we detected significantly elevated presence of NK cells in GBM tumors in brains of treated mice, which correlated to higher chemokine levels (**Fig.1L** and **M**). **Conclusions:** We built a novel, sophisticated combinatorial platform for GBM: a multifunctional immunotherapy based on genetically-engineered, human NK cells bearing multiple antitumor functions, including local tumor responsiveness, that addresses, for the first time, key drivers of GBM resistance to therapy: antigen escape, immunometabolic reprogramming of immune responses, and poor immune cell homing. **References** [1] Pombo Antunes AR, Scheyltjens I, Duerinck J, Neyns B, Movahedi K, Van Ginderachter JA. Understanding the glioblastoma immune microenvironment as basis for the development of new immunotherapeutic strategies. Elife. 2020;9:e52176.

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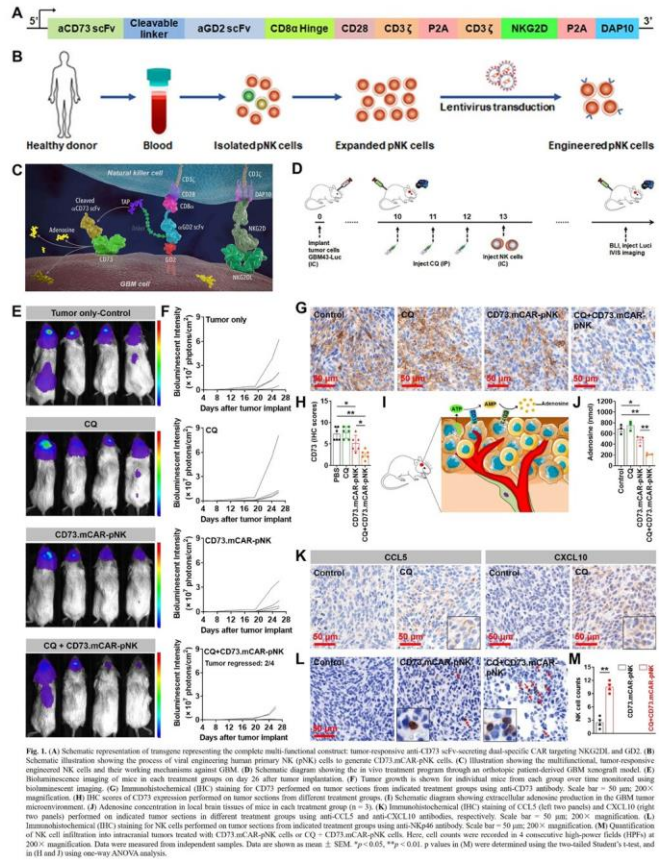

Fig. 1. (**A**) Schematic representation of transgene representing the complete multi-functional construct: tumor-responsive anti-CD73 scFv-secreting dual-specific CAR targeting NKG2DL and GD2. (**B**) Schematic illustration showing the process of viral engineering human primary NK (pNK) cells to generate CD73.mCAR-pNK cells. (**C**) Illustration showing the multifunctional, tumor-responsive engineered NK cells and their working mechanisms against GBM. (**D**) Schematic diagram showing the in vivo treatment program through an orthotopic patient-derived GBM xenograft model. (**E**) Bioluminescence imaging of mice in each treatment groups on day 26 after tumor implantation. (**F**) Tumor growth is shown for individual mice from each group over time monitored using bioluminescent imaging. (**G**) Immunohistochemical (IHC) staining for CD73 performed on tumor sections from indicated treatment groups using anti-CD73 antibody. Scale bar = 50 μm; 200× magnification. (**H**) IHC scores of CD73 expression performed on tumor sections from different treatment groups. (**I**) Schematic diagram showing extracellular adenosine production in the GBM tumor microenvironment. (**J**) Adenosine concentration in local brain tissues of mice in each treatment group (n = 3). (**K**) Immunohistochemical (IHC) staining of CCL5 (left two panels) and CXCL10 (right two panels) performed on indicated tumor sections in different treatment groups using anti-CCL5 and anti-CXCL10 antibodies, respectively. Scale bar = 50 μm; 200× magnification. (**L**) Immunohistochemical (IHC) staining for NK cells performed on tumor sections from indicated treatment groups using anti-NKp46 antibody. Scale bar = 50 μm; 200× magnification. (**M**) Quantification of NK cell infiltration into intracranial tumors treated with CD73.mCAR-pNK cells or CQ + CD73.mCAR-pNK cells. Here, cell counts were recorded in 4 consecutive high-power fields (HPFs) at 200× magnification. Data were measured from independent samples. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$. p values in (M) were determined using the two-tailed Student's t-test, and in (H and J) using one-way ANOVA analysis.

151. ADCLEC.syn1 Is a Novel Combinatorial CAR Platform for Enhanced Therapeutic Index in AML

Sascha Haubner, Jorge Mansilla-Soto, Sarah Nataraj, Jae Park, Xiuyan Wang, Isabelle Rivière, Michel Sadelain

Center for Cell Engineering and Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY

Relapsed/refractory (r/r) AML is associated with very poor prognosis. The only curative option to date is allogeneic stem cell transplantation which is limited due to high treatment-related toxicity and therapeutic failure, creating a high medical need for potent yet tolerable novel therapies. CAR therapy has high potential for successful application beyond CD19-positive B cell malignancies, however suitable CAR targets in AML still need to be identified. Integrating proteomic and transcriptomic target expression data we have previously discovered n=24 CAR candidate targets with a favorable profile in AML and normal tissues and provided a rationale for several combinatorial CAR approaches in AML (Perna et al. Cancer Cell 2017). We have further validated

lymphopenia resulting in inadequate immune intratumoral trafficking all contribute to glioblastoma (GBM) being highly resistant to therapy [1]. Though intratumoral presence of NK cells is beneficial to GBM patients, current GBM immunotherapies have struggled to overcome these challenges and demonstrate sustained clinical improvements in patient overall survival (OS). As a result, new immunotherapeutic approaches for this deadly cancer that are able to address all three of these critical hurdles are needed. **Methods:** We generated multifunctional human NK (CD73.mCAR-pNK) cells that express a dual-specific CAR redirected against ligands for NKG2D and GBM-associated GD2 receptors, and a third functional moiety that can be activated in the GBM TME to address immunometabolic

favorable target pairs among our 24 candidates, based on a multimodal in-depth analysis including multiparameter spectral flow cytometry of primary r/r AML and normal bone marrow samples, along with normal tissue immunohistochemistry studies and mass-spectrometry. We focus here on ADGRE2 and CLEC12A, two cell surface molecules highly co-expressed in AML but with largely non-overlapping expression profiles in normal tissues. We hypothesized that rational combinatorial CAR design targeting both ADGRE2 and CLEC12A enhances anti-leukemic efficacy without cumulating potential on-target/off-tumor toxicity. Using a bicistronic gamma-retroviral vector, we screened different combinatorial CAR formats targeting ADGRE2 and CLEC12A. Specific to the combined target expression profile in malignant versus normal cells, we fine-tuned both scFv affinities considering total avidity, eventually achieving thresholds for mediating cytolysis in the context of optimized CD3 zeta signaling. In addition, we further optimized the chimeric receptor combination by evaluating different hinge/ transmembrane and costimulatory domains. To provide a platform for identification of the ideal combinatorial CAR design, we established in-vitro and in-vivo models based on a human AML cell line with up- or down-regulated antigen levels of ADGRE2 and CLEC12A to mimic both AML antigen-low escape and toxicity to normal cells. Ultimately, using therapeutically relevant T cell doses we were able to identify a combinatorial CAR format that allowed complete and durable AML remission across relevant target levels while sparing cell line clones representing normal cells.

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152. Systemic *Hps1* Gene Augmentation Prevents Pulmonary Manifestations in a Mouse Model of Hermansky-Pudlak Syndrome

Shachar Abudi[1,2], Marina Zieger[3], John D. Burke[1], Lisa J. Garrett[4], Yair Anikster[2,5], Bernadette R. Gochuico[1], William A. Gahl[1,6], Christian Mueller[3], May C. Malicdan[1,6]

[1]Human Biochemical Genetics Section, NHGRI, NIH, Bethesda, MD,[2]Tel Aviv University, Tel Aviv, Israel,[3]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[4]Transgenic Mouse Core, NHGRI,
NIH, Bethesda, MD,[5]Sheba Medical Center, Ramat Gan, Israel,[6]NIH UDP, NHGRI, NIH, Bethesda, MD

Mutations in *HPS1* cause Hermansky-Pudlak syndrome type 1 (HPS1), an autosomal recessive disorder manifesting with oculocutaneous albinism, a bleeding diathesis, and a highly penetrant and lethal form of pulmonary fibrosis (HPSPF). A prominent finding in the lungs of HPSPF patients is the presence of enlarged and foamy type II alveolar cells (AECII), which are thought to have critical roles in alveolar homeostasis. Alteration in the function of AECII cells have been shown to promote dysregulated repair and pathogenic activation of fibroblasts, ultimately leading to fibrosis. There is no FDA-approved treatment for HPSPF; the identification of effective therapy has been hindered by the lack of preclinical models that represent the human HPS genotype and phenotype. Because *HPS1* mutations are lossof-function, we hypothesized that introduction of a normal copy of *HPS1* could be a strategy for treatment. With the goal to establish gene therapy for HPS-1, we systemically administered adeno-associated virus (AAV) harboring the open reading frame of the murine *Hps1* to a novel *Hps1* knockout mouse that we generated.We deleted *Hps1* in mice by using CRISPR-Cas9 ($Hps1^{delta/delta}$). These mice recapitulated human HPS-1 phenotypes, and presented with enlarged and foamy AECII, increased susceptibility to bleomycin-induced PF, reduced pigmentation, and a bleeding diathesis. Molecularly these mice show no *Hps1* mRNA expression in their lungs and other tissues. To determine which AAV serotype is suited to target AECII, we injected AAV5 or AAV8 harboring GFP to one-day old via the facial vein. We performed *in situ* hybridization of RNA, immunohistochemistry of GFP protein and molecular quantification. Our results reveal that AAV5 and AAV8-GFP are highly expressed in lung cells, including AECII, after 1 month of augmentation.For *in vivo* gene augmentation, we injected systemically AAV5 or AAV8 harboring murine *Hps1* to one-day old mice. Phenotypic characterization included molecular analysis, examination of lung pathology, and measurement of lung physiology. Six months after gene augmentation mice given AAV5Hps1 or AAV8-Hps1 revealed an increase of about 80% in *Hps1* mRNA in their lungs compared to lungs of untreated mice that did not express *Hps1* mRNA. More importantly, histological analysis showed improvement of the lung phenotype including reduction in the size and number of enlarged and foamy AECII (Figure 1), which correlated with higher *Hps1* expression. Taken altogether, our results show that gene augmentation of AAV can prevent the pulmonary manifestations in our *Hps1* knockout mouse and can be used in designing therapeutic options for HPSPF.

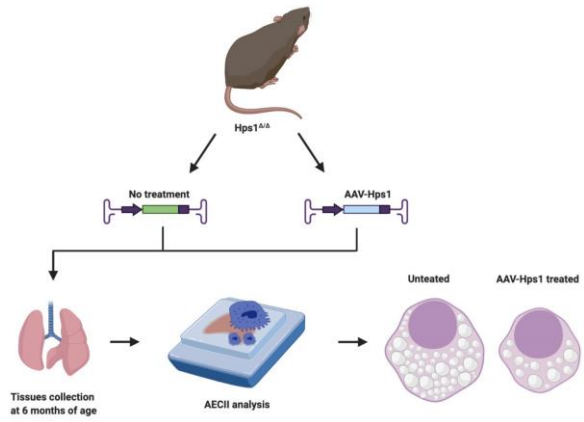

Figure 1. Schematic presentation of approach to gene augmentation.
AAV5 or AAV8 encoding *Hps1* were injected into $Hps1^{delta/delta}$ mice. Tissues were collected and analyzed after 6 months. Lung histology of $Hps1^{delta/delta}$ untreated mice reveals enlarged and foamy type II alveolar epithelial cells (AECII). Mice that were transduced with AAV5 or AAV8 *Hps1* had smaller and less foamy AECII.

153. Generation of a Human 3D Lung Model for Therapeutic Gene Editing in Surfactant Protein B Deficiency

Helena C. M. Meyer-Berg, Stephen C. Hyde, Deborah R. Gill

Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

Surfactant protein B (SP-B) deficiency is a rare autosomal recessive disease of the lung leading to severe respiratory distress that is fatal within the first months of life. Conventional therapies such as steroids or surfactant replacement therapy are ineffective in these individuals. Gene therapy has the potential to treat SP-B deficiency by restoring surfactant homeostasis. Cross-species variation can hamper gene therapy development, because of variable expression of viral entry receptors crucial for (e.g. rAAV) vector entry. Moreover, genomic differences complicate testing of targeted gene editing tools in non-human models. We established a human model of SP-B deficiency to address these complications, choosing a human 3D-lung model generated from the human embryonic stem cell line RUES2. These lung bud organoids (LBOs), described by Chen and colleagues [Nat Cell Biol, (2017) 19: 542], exhibit a bias towards generation of lung parenchyma, especially surfactant-producing ATII cells. We previously used this model to screen rAAV serotypes suitable for transduction of the human parenchyma and identified rAAV6.2 as a promising candidate, concluding that LBOs are a suitable lung gene therapy model [Stem Cell Res Ther 2020 11: 448]. To grow SP-B deficient LBOs, RUES2 embryonic stem cells were gene edited to achieve the 121ins2 (C > GAA) mutation in SFTPB - the most common mutation in SP-B deficiency (ca. 66% of cases). HDR gene editing was performed by electroporation of Cas9 RNP (Synthego) and ssDNA donor. Because of the low efficiency of HDR editing, electroporation was first optimised. The highest levels of HDR gene editing were achieved using the CA137 electroporation programme (Lonza), with up to 89 % indel formation and 59 % knock-in achieved as determined by ICE analysis. After cloning and screening of 58 clones, 53 % were identified as 121ins2 edited via restriction fragment length polymorphism analysis. Next, 14 RUES2 121ins2-edited clonal lines were confirmed by Sanger sequencing reactions, twice after isolation and twice during clonal expansion. Two clones were taken forward for further characterisation. A normal karyotype was confirmed for both via KaryoStat microarray and the retention of stem cell status was examined for both lines by immunocytochemistry for markers SOX2, OCT4, TRA-1-60 and SSEA4. LBOs were grown from the parental line and from one of the 121ins2 edited clones. Firstly, endoderm was induced and induction efficiency determined by flow cytometry. The parental cell line and the edited clone were 90.2 % and 90.5 % double positive for the endoderm markers CXCR4 and c-Kit, respectively. During anterior foregut endoderm induction, expression of FOXA2 was confirmed at similar levels in both cell lines with immunocytochemistry. Finally, lung progenitor organoids were placed in Matrigel for maturation and branching. The morphology was documented over the entire organoid generation and at no point were differences between cell lines observed. Buds developed within 2 weeks, with similar budding efficiency observed. On day 77 of maturation, Western blot analysis indicated expression of SP-B in the wild-type organoids and its absence in the 121ins2 edited LBOs. In summary, we have generated a human embryonic stem cell line to model the SP-B deficient lung, with opportunity to screen for gene therapies or therapeutic gene editing strategies, in an effort to accelerate translational research.

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154. Vectored Immunoprophylaxis for COVID-19 (COVIP)

Yue Du[1], Kamran Miah[1], Habib Omar[1], Helena Meyer-Berg[1], YanQun Wang[2], JinCun Zhao[2], Stephen Hyde[1], Deborah Deborah[1]

[1]Nuffield Department of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom,[2]State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital, Guangzhou, China

A quest for effective therapeutics and prophylactic strategies for COVID-19 pandemic is still an active pursuit. Protein-based therapeutics, such as monoclonal antibodies (mAbs) that target epitopes on the SARS-CoV-2 Spike (S) protein represent a novel approach, but such therapeutics typically have short half-lives, necessitating repeated delivery. A more promising strategy against SARS-CoV-2 infections is to use vector-mediated immunoprophylaxis (VIP) by directing the sustained production of neutralising mAb into serum or in a localized fashion. Among multiple recombinant viral vectors, our preferred vector is based on a third-generation, selfinactivating simian immunodeficiency virus (rSIV) pseudotyped with the Fusion and Haemaglutinin-Neuraminidase surface glycoproteins from Sendai virus (rSIV.F/HN), which directed a sustained expression of broadly neutralising mAb to confer completely protection against supra-lethal influenza infection (Tan 2020 Thorax 2020;75:1112). As a comparison, we also included recombinant Adeno-associated virus (rAAV) vector 9 and 8 serotypes, which are gold standard to offer VIP against pathogens of importance.Since the laboratory BALB/c mice are not naturally susceptible to Coronavirus, we provided human ACE2 (hACE2) in trans via rAAV9 vector to facilitate SARS-CoV-2 pseudovirus (encoding luciferases; S-LV.luciferase) entry in lungs. Intranasal (IN) delivery of S-LV to the hACE2-expressing mice resulted in dose-dependent luciferase expression, which peaked at peaked at 7 days post-delivery (8.72E4±4.14E4 p/s/cm2/sr, Average Activity Radiance). Ultimately, we utilised hACE2-expressing murine model and S-LV as a means to investigate a novel VIP for COVID-19 (COVIP) strategy.The sequence for anti-SARS-CoV-2 monoclonal antibody (mAb) NC0321, isolated from the PBMCs of a COVID-19 convalescent patient, was inserted into the rSIV.F/HN.hCEF, rAAV. hCEFI and rAAV.CASI vectors. To test prophylaxis, 1e11 GC AAV9 or 5e8 TU rSIV.F/HN mAb vector were co-administrated in a single IN administration with 1e11 GC rAAV9.hACE2; 1e11 GC of rAAV9 mAb vector was delivered by intramuscular injection and followed by an IN delivery of rAAV9.hACE2 under the same anaesthesia conditions. The NC0321 IgG expression level was increased from day 7 to day 14, reaching a plateau at day 14 onwards. At day 28 post-delivery, IgG expression level was about 0.5,1 and 5 μg/ml for rSIV.F/HN, rAAV9, and

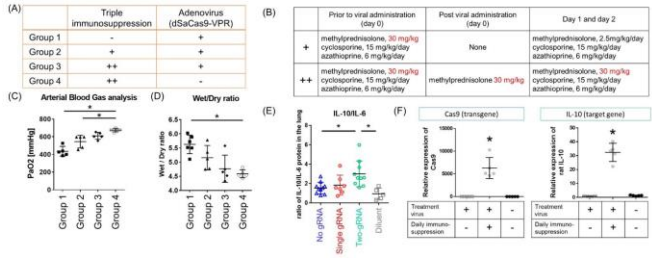
rAAV8 vectors expressing NC0321, respectively. In all three cases, NC0321 IgG expression in sera was significantly higher than that in naïve mice (****, *p<0.001*). At 28 days post-delivery, the IgG expression level in the mouse epithelial lining fluid was about 5, 50 and 20 μg/ml for rSIV.F/HN, rAAV9, and rAAV8 vectors expressing NC0321, respectively. Encouragingly, on maximal challenge with S-LV. luc to hACE2-expressing mice, luciferase expression was significantly reduced in mice intranasally dosed with rSIV.F/HN and rAAV9 vector expressing NC0321 compared with an isotype control (**, *p=0011* or *0.0052*, respectively; AUC of the average luminescence). Interestingly, rAAV8-mediated expression of NC0321 conferred no significant protection.Here we compare different vector platforms and delivery routes for SARS-CoV-2 specific antibody gene transfer based on a hACE2 murine model. Our study indicates that vector delivery of mAb by direct lung inhalation route is efficient to confer protection against SARS-CoV-2 mimic. This COVIP strategy may offer protective immunity for vulnerable individuals unable to mount an effective immunological response for COVID-19.

155. Impact of Transplant Immunosuppression on In Vivo Lung-Selective CRISPR/Cas9 Therapeutics for Lung Transplantation

Kumi Mesaki, Stephen Juvet, Zehong Guan, Jim Hu, Alan Davidson, Marcelo Cypel, Mingyao Liu, Shaf Keshavjee

University Health Network, Toronto, ON, Canada

Background: Donor lung gene modification using CRISPR/Cas9 holds promise in lung transplantation (LTx). Immunologic enhancement of donor lungs by epigenetic or genetic modification using CRISPR/Cas9 could address the shortage of donor organs, improve outcomes, and eventually eliminate the need for life-long systemic immunosuppression. For CRISPR/Cas9 therapeutics to modulate a whole donor organ, efficient delivery is critical to achieve the therapeutic effects. Adenoviral vectors provide high efficacy with rapid expression and have sufficient capacity to deliver the Cas9 and guide RNAs (gRNAs) in a single package. Nonetheless, adenoviruses are known to have significant immunogenicity, particularly in the lung - an organ vulnerable to inflammation and injury. Consequently, expression of transgenes after adenoviral delivery is transient. Further, Cas9 is itself immunogenic, thus creating an additional potential barrier to clinical translation of CRISPR/Cas9 therapeutics with adenoviral vectors. Anti-rejection immunosuppression required in LTx can be utilized to minimize vector-related inflammation. Furthermore, viral delivery to the organ ex vivo, prior to host exposure to the graft further facilitates this process. This has made the possibility of gene therapy more promising. We therefore hypothesized that the use of transplant immunosuppression could permit effective CRISPR/Cas9 therapeutics in vivo in the lung. **Methods**: Adenovirus expressing a Cas9 activator (dSaCas9VPR) was trans-bronchially delivered to Lewis rats at a high dose (2.5E8 PFU/rat). The effect of standard triple immunosuppression (methylprednisolone, cyclosporine, and azathioprine) with or without additional methylprednisolone on vector-related inflammation was assessed in four groups (Fig A, B) after 72 hours. Next, to elucidate the impact of immunosuppression on the persistence of epigenome editing, adenovirus expressing Cas9 activator and gRNAs was delivered to activate the IL-10 gene at low dose (1E8 PFU/rat), and assessed after day 14 with or without daily immunosuppression. **Results**: Delivery of high dose adenovirus expressing Cas9 activator without immunosuppression (Group 1) worsened oxygenation and lung edema: PaO2 (435±55 vs 674±20, p<0.0001), wet/dry ratio (5.63±0.33 vs 4.59±0.14, p=0.0013) compared to the diluent group (Group 4, Fig C, D). Triple immunosuppression (Group 2) improved oxygenation compared to the no immunosuppression group (Group 1). Additional steroid administration (Group 3) further improved the condition of the lung leading to PaO2 (610±38, p=0.35) and wet/dry (4.77±0.48, p>0.99) ratios comparable to the diluent (control) group (Group 4). Adenovirus expressing two gRNAs induced IL-10 gene expression with minimal inflammation at low dose, as shown by a significantly increased IL-10/IL-6 protein ratio in the lung (Fig E). Daily immunosuppression sustained IL-10 gene activation until day 14 (relative expression; 32.3±6.5 vs 0.67±0.14, p<0.0001, Fig F) as well as Cas9-expression and viral DNA, suggesting immunosuppression inhibited elimination of the transduced cells. **Conclusion**: Transplant immunosuppression ameliorates inflammation and prolongs the effect of CRISPR/Cas9 epigenome editing with adenovirus in the lung. This study illustrates a strategy for effective gene editing by managing vector-related inflammation in an in vivo CRISPR/Cas9 therapeutics.



156. First Proof-of-Concept of miQURE™ Based Gene Targeting in the Liver: Lipid Lowering and Atherosclerosis Suppression by AAV-miQURE™-Mediated ANGPTL3 Targeting

Vanessa Zancanella[1], Astrid Valles-Sanchez[1], Carlos Vendrell Tornero[1], Maroeska Oudshoorn-Dickmann[1], Hendrina Wattimury[1], Kristel van Rooijen[1], Mark van Veen[1], Monika Golinska[1], Elsbet J. Pieterman[2], Nanda Keijzer[2], Hans M. G. Princen[2], Geurt Stokman[2], Martin de Haan[1], Ying Poi Liu[1]

[1]uniQure Biopharma B.V., Amsterdam, Netherlands,[2]TNO Metabolic Health Research, Leiden, Netherlands

We have developed a proprietary, next-generation miQURE™ technology, where a transcript encoding artificial microRNAs (miRNAs) is packaged into adeno-associated viral (AAV) vectors for mRNA silencing. Our AAV5-miQURE™ targeting mutant huntingtin in the brain is currently being tested in a phase 1/2a clinical study in Huntington Disease patients. In the current study, we have used Dyslipidemia as indication to investigate the potency of AAV-based miQURE™ to lower Angiopoietin-like 3 (ANGPTL3) in the liver. ANGPTL3 loss-of-function genetic variants have been associated with low levels of plasma

triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, and a decreased risk of cardiovascular diseases. Pharmacological inhibition of ANGPTL3 mimics the phenotype of individuals carrying mutations impairing ANGPTL3 and lowers plasma lipids in mice, monkeys and humans. After *in vitro* testing, the most promising miQURE™ candidates were tested for their efficacy to lower ANGPTL3 mRNA and ANGPTL3 plasma levels in wild-type and transgenic mice with a humanized lipoprotein metabolism (APOE*3-Leiden.CETP). The lead candidate resulted in wild-type mice in a dose-dependent decrease in ANGPTL3 mRNA expression (up to ~77%) and a subsequent up to ~90% reduction

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in ANGPTL3 plasma levels. APOE*3-Leiden.CETP mice received AAV-miANGPTL3 treatment with or without the standard of care treatment, statin (atorvastatin). AAV5-miANGPTL3 delivery to the liver resulted in a significant and sustained reduction in triglycerides and plasma total cholesterol, whereas the levels in the vehicle and control scrambled miRNA groups remained unchanged. Furthermore, a significant decrease in total atherosclerotic lesion area could be observed in the AAV-miANGPTL3 treated group (58% compared to scrambled miRNA). Treatment with AAV-miANGPTL3 was associated with maintenance of an non-diseased plaque phenotype and reduction in the lesion severity. A further reduction in total cholesterol and triglycerides exposure and in the total atherosclerotic lesion area was observed in the combination treatment with atorvastatin. To provide proof of concept of liver directed miANGPTL3 expression, a non-human primates study was conducted. In this study, the animals received a high caloric diet to induce hyperlipidemia. Similar to the mouse study, the main goal was to assess miRNA, mRNA and relevant plasma lipid markers. The AAV-miANGTPL3 treatment was welltolerated in all animals during the in-life phase of 22 weeks. However, the individual animal response on the high calory diet varied greatly, resulting in variable data. Nevertheless, preliminary results suggest that the AAV5-miANGTPL3 group showed a slight lowering in total cholesterol and triglycerides. In conclusion, we here report the first proof-of-concept of a miQURE™-based approach in the liver. The combination of tolerability of miQURE ™ expressed in the liver of non-human primates and proof-of-concept in mice supports the continuation of its development for liver-directed indications and translation in the clinic.

157. Abstract Withdrawn

Clinical Trials and Advanced Preclinical Studies for Neurologic Diseases

158. Electroporation Mediated Gene Transfer of MRCKα to the Lungs of Mice Effectively Treats Pre-Existing Acute Lung Injury

Jing N. Liu[1], Michael Barravecchia[2], David A. Dean[2]

[1]The Cellular and Molecular Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY,[2]Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, NY

Acute Lung Injury (ALI) and its more severe form Acute Respiratory Distress Syndrome (ARDS) are severe conditions characterized by alveolar fluid accumulation and insufficient gas exchange, ultimately leading to acute respiratory failure. Both impaired alveolar fluid clearance (AFC) and a disrupted alveolar-capillary barrier contribute to the pathogenesis of ALI/ARDS. Most attempts at therapy in the past have focused on enhancing AFC, but repairing the alveolarcapillary barrier is likely also needed for effective treatment. We previously have shown that overexpression of the β1 subunit of the Na^+, K^+-ATPase (β1- Na^+, K^+-ATPase) increases AFC in lungs of multiple species. We also have found that electroporation-mediated gene delivery of the β1- Na^+, K^+-ATPase rescues lipopolysaccharide (LPS) induced ALI by upregulating tight junction (TJ) proteins and pulmonary barrier function, demonstrated by decreased lung permeability, total protein and cellularity in bronchoalveolar lavage (BAL) fluid, and improved overall outcome of lung injury. While studying how the β1- Na^+, K^+-ATPase increases lung barrier, we identified MRCKα (CDC42 binding protein kinase alpha) as an interacting partner of the β1 subunit. Previous data from our lab indicate that MRCKα mediated β1's upregulation of TJ proteins and epithelial barrier integrity in cultured cells. However, it is unknown whether MRCKα can upregulate pulmonary barrier function and treat LPS induced ALI *in vivo*. Understanding this question would help to determine the therapeutic potential of MRCKα for ARDS. Plasmids expressing MRCKα or β1- Na^+, K^+-ATPase were delivered individually or in combination by aspiration and transthoracic electroporation to mice which had been pre-injured by LPS intratracheal administration 24 hours earlier. Two days after gene delivery, various endpoint assays were performed to evaluate lung edema, permeability, inflammation and histological injury. We found that overexpression of MRCKα alone attenuated LPSincreased edema, lung leakage, BAL cellularity and protein concentration, restored TJ protein expression, and improved overall outcome of lung injury, similar to gene transfer of the β1- Na^+, K^+ATPase. However, we found that unlike β1- Na^+, K^+-ATPase, gene transfer of MRCKα alone did not enhance AFC. These results indicate that MRCKα could benefit the pre-injured lungs by reducing pulmonary edema, restoring lung barrier function and reducing inflammation. Moreover, they also suggest that improving barrier function alone may be of equal or

even more benefit than improving AFC in order to treat ALI/ARDS. (Supported by: NIH grants HL120521, HL131143, and HL138538 and an AHA predoctoral fellowship (JL))

Clinical Trials and Advanced Preclinical Studies for Neurologic Diseases

159. Gene Therapy Candidate for Metachromatic Leukodystrophy (MLD):

Summary of Preclinical *In Vivo* Data Following an Intravenous Delivery of HMI-202

Jacinthe Gingras, Thia St-Martin, Katie Gall, Tania A. Seabrook, Jason Lotterhand, Israel Rivas, Nancy Avila, Michael Mercaldi, Jennifer Newman, Shiva Krupa, Teresa Wright, Omar Francone, Albert Seymour
Homology Medicines Inc, Bedford, MA

Metachromatic leukodystrophy (MLD) is an inherited autosomal recessive lysosomal storage disorder (LSD) with a great unmet medical need. This fatal neurodegenerative LSD occurs in three forms: late infantile (prevalence of 1 in 40,000), juvenile, and adult. The late infantile and juvenile forms represent the majority of the MLD patients where mortality at 5 years is estimated at 75% and 30%, respectively. MLD is most commonly caused by mutations in the *ARSA* gene and patients suffering from the disease are deficient in arylsulfatase-A (ARSA) enzyme activity. The disease is characterized by accumulation of sulfatides to supraphysiologic and toxic levels in the peripheral organs and nervous system. In the brain, excess sulfatides lead to the destruction of myelin, a key protective layer of the nerve fibers that enhances propagation of action potentials. Herein, we report preclinical gene therapy data where a single intravenous (IV) dose of HMI-202 (AAVHSC15-human-*ARSA* (h*ARSA*)) crosses the blood-nerve- and blood-brain-barriers (BNB and BBB) in juvenile non-human primates (NHP) and in the *Arsa* KO murine model of MLD. In the HMI-202treated adult *Arsa* KO mice, hARSA expression patterns are nearly identical to that of murine Arsa (mArsa) distribution in the nervous system of wild type age-matched littermates, in both neuronal and glial cellular profiles. In HMI-202-treated adult *Arsa* KO mice, we show a dose-response relationship in hARSA enzyme activity, transcript, and vector genomes in the central nervous system (CNS). As early as 1 week following administration (earliest time-point of collection), near-normal human adult levels of hARSA activity are detected in the CNS of HMI-202-treated adult *Arsa* KO mice, and levels are sustained at or above normal adult human brain levels throughout the study (52 weeks post-dose). Similarly, hARSA enzyme activity is detected 1 week post-dose in the CNS of *Arsa* KO neonates and is sustained out to 12 weeks post-dose (end of study). Furthermore, we demonstrate modulatation of key biochemical markers in the CNS, including murine neuronal sulfatides, myelin and lymphocyte (MAL) transcript, lysosomal-associated membrane protein-1 (LAMP-1), and glial fibrillary acidic protein (GFAP) levels in HMI-202-treated *Arsa* KO mice. Lastly, using the rotarod assay, we demonstrate a functional motor benefit in HMI-202-treated *Arsa* KO mice dosed prior to the detectable accumulation of CNS neuronal sulfatides (~2 months of age). In summary, a single-IV dose of HMI-202 crossed the BNB and BBB in lower (mice) and higher (NHP) species. In addition, the ability to achieve hARSA enzyme activity levels at or above normal human adult brain levels, rapid onset of expression, durability, broad biodistribution, modulation of key biomarkers, and functional motor benefit in a murine MLD disease model was demonstrated. These preclinical data from IND-enabling studies continue to support the further optimization and development of HMI-202 as a gene therapy for the treatment of MLD.

160. Gene Replacement Therapy for Angelman Syndrome

Justin Percival, Kasturi Sengupta, Long Le, Khalid Arhzaouy, Heather Born, Elizabeth Buza, Cecilia Dyer, James M. Wilson
Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Angelman syndrome (AS) is a rare neurodevelopmental disorder affecting approximately half a million individuals worldwide. AS is characterized by intellectual and physical disability, seizures, impaired sleep, and gut dysfunction. Many of these deficits result from a loss of the maternally inherited ubiquitin protein ligase E3A (*UBE3A*) allele. At present, therapeutic options for AS are limited. Adeno-associated virus (AAV)-based gene replacement therapy represents a promising strategy for restoring *UBE3A* isoform expression and mitigating AS severity. However, the *UBE3A* gene encodes three isoforms, and it is currently unclear which *UBE3A* isoform is the most effective candidate. We addressed this uncertainty by comparing the efficacy of AAV vectors delivering codon-optimized human *UBE3A* isoforms in a mouse model of AS. Western blotting and immunohistochemical analyses indicated that intracerebroventricular injections of AAV-UBE3A human isoform 1 and 2 vectors into neonatal control and AS mice resulted in robust protein expression. Isoform 1 replacement significantly improved gait, nest-building ability, and motor coordination in a dose-dependent manner in AS mice. In contrast to isoform 1, isoform 2 further impaired nest-building ability and motor coordination in AS mice. Ongoing toxicology studies in nonhuman primates suggest that a high dose of AAV-UBE3A isoform 1 vector injected into the cisterna magna has no significant adverse effects. Taken together, these data indicate that the AAV-UBE3A isoform 1 vector is the most therapeutically effective option with a promising safety profile. These preclinical findings represent an important step forward in the development of gene replacement therapy for AS.

161. An AAV-miRNA for Androgen Receptor Knockdown in Spinal and Bulbar Muscular Atrophy

Eileen Workman, Julia Johansson, Mariya Kostiv, Christian Hinderer, James M. Wilson
Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Introduction: Spinal and bulbar muscular atrophy (SBMA) is an X-linked, slowly progressive motor neuron disease caused by a polyglutamine (CAG) expansion tract within exon 1 of the androgen receptor (AR). The expansion results in the nuclear aggregation of the

AR protein, which then causes motor neuron degeneration almost exclusively in males due to androgen-mediated activation of toxicity. To date, no effective treatment has been approved for SBMA. Since knockdown of the androgen receptor in neurons does not appear to cause adverse effects, lowering AR levels in SBMA is an attractive strategy for treating the disease. Our gene therapy approach involves delivering an AAV vector expressing an miRNA targeting the AR to motor neurons. **Results:** We screened for artificial miRNAs

targeting the AR in cell culture and identified a candidate miRNA. Artificial miRNAs were cloned into a miR-155 backbone with a cytomegalovirus promoter and transfected into HEK293 cells. Using qPCR and Western blot analysis, we identified an AR-targeting candidate miRNA, GTP-miR001, which shares homology in mouse, human, and rhesus macaque. GTP-miR001 was able to knock down up to 60% of AR expression in HEK293 cells. The candidate miRNA was packaged into an AAV. PHP.eB vector for *in vivo* testing in wild-type C57BL/6J mice. It was determined that IV injection of AAV.PHP.eB.GTP-miR001 at 3e11 was sufficient to achieve greater than 50% knockdown of the androgen receptor in the mouse brain and 70% knockdown in the spinal cord. To evaluate the potential efficacy of GTP-miR001 for the treatment of SBMA, we tested an AAV serotype hu68 vector expressing the miRNA in AR97Q SBMA transgenic mice. These mice express high levels of the human AR with a pathogenic repeat expansion and display a very severe SBMA phenotype with progressive hindlimb weakness that ultimately results in death. Males have a median survival of 91 days and females have a median survival of 184 days. Intravenous administration of the AAVhu68.GTP-miR001 vector in 3- or 5-week-old mice resulted in efficient knockdown of the AR, with modest improvements in survival and motor phenotype that correlated with the degree of AR suppression. Treating AR97Q mice as neonates yielded robust improvements in weight gain, motor phenotype, and survival, indicating the necessity of early intervention in this rapidly progressive mouse model. **Conclusions:** We have identified a candidate AAV vector expressing an artificial miRNA for the treatment of SBMA. This vector was capable of knocking down AR protein expression *in vitro* and *in vivo*. Further, we were able to achieve improvement of the phenotype and survival of AR97Q SBMA transgenic mice when subjects were injected at the neonatal stage. Ongoing studies include the determination of vector toxicity and knockdown of AR protein in nonhuman primates.

162. AXO-AAV-GM1 for the Treatment of GM1 Gangliosidosis: Preliminary Results from a Phase I-II Trial

Cynthia J. Tifft[1], Precilla D'Souza[1], Jean Johnston[1], Maria Acosta[1], Caroline Rothermel[1], Audrey Thurm[2], Ajith Karunakara[3], Benjamin Thorp[3], Peter Ross[3], John Jameson[3], Toby Vaughn[3], Donna Valencia[3], Erika De Boever[3], Gavin Corcoran[3]

[1]Office of the Clinical Director, NHGRI, National Institutes of Health, Bethesda, MD,[2]National Institute of Mental Health, National Institutes of Health, Bethesda, MD,[3]Sio Gene Therapies, New York, NY

GM1 gangliosidosis is a rare, inherited neurodegenerative disorder caused by mutations in the *GLB1* gene which encodes the lysosomal hydrolase β-galactosidase (β-gal). The resulting enzyme deficiency leads to a toxic accumulation of GM1 ganglioside, predominantly in the central nervous system (CNS) where its rate of synthesis is the highest, but also in peripheral tissues. GM1 gangliosidosis is uniformly fatal, and there are no disease-modifying treatments currently available. As this is a monogenic disorder, it is an ideal target for gene therapy to deliver β-gal to the CNS and periphery, with resulting potential to halt further neurodegeneration, restore function and ameliorate symptoms. This is a preliminary analysis from an ongoing open-label, single-arm, Phase I-II trial (ClinTrials.gov, NCT03952637) in which subjects with a confirmed genetic and biochemical diagnosis of GM1 gangliosidosis were treated with AXO-AAV-GM1 (AAV9-GLB1), an investigational gene therapy utilizing an adeno-associated virus (AAV9) vector to deliver a functional copy of the *GLB1* gene. All subjects received 1.5 $x10^{13}$ vg/kg of AXO-AAV-GM1 administered via intravenous infusion and immune modulation with rituximab, sirolimus and glucocorticoids. The primary endpoint of the trial is safety/tolerability, secondary efficacy endpoints include Vineland-3, brain MRI, motor function and disease severity. Biomarkers of disease progression or stabilization (βgal activity and GM1 ganglioside in serum and CSF) are also assessed. Six-month follow-up data are presented from 4 subjects with the late infantile (Type IIa) form and one with juvenile (Type IIb) disease. AXO-AAV-GM1 was generally safe and well-tolerated and there have been no serious adverse events (SAEs) related to gene therapy. One SAE was described: a single subject experienced bacterial sepsis due to a PICC line infection, which was considered to be unrelated to the investigational product, and which resolved following line removal and administration of IV antibiotics. The most common adverse events were considered mild to moderate. Four subjects had AST elevations that were considered to be adverse events. None required clinical intervention or had associated clinical sequelae. There were no other adverse events indicative of impaired liver function including serum bilirubin, GGT, and ALT. Serum β-gal enzyme activity was sampled at 9 distinct post-baseline timepoints between Day 7 and Month 6. The mean increase from baseline ranged from 71-138% across the 9 time points. At Month 6, serum enzyme activity was sustained with an increase from baseline ranging from 33%-128% across the 5 subjects. Subjects were assessed by multiple measures of neurodevelopment including the Vineland Adaptive Behavior Scales 3rd Edition (VABS-3), Upright and Floor Mobility Score, and Clinical Global Impression (CGI). All 5 subjects demonstrated disease stability at 6 months post-treatment as assessed by VABS-3 Growth Scale Value scores, Upright and Floor Mobility Score, and CGI relative to baseline values. Per protocol, brain MRIs were not collected for the 6-month follow-up analysis but will be completed at later timepoints. We will continue to follow-up these subjects and are now evaluating additional subjects receiving 4.5 $x10^{13}$ vg/kg dose of AXO-AAV-GM1.

163. AXO-Lenti-PD Gene Therapy for Parkinson's Disease: Efficacy, Safety, and Tolerability Data from the Second Cohort in Open-Label Dose Evaluation Study SUNRISE-PD at 6 Months Post Administration

Gavin Corcoran[1], Ajith Karunakara[1], Ben Vaughn[2], Elimor Brand-Schieber[1], Thomas Foltynie[3], Roger A. Barker[4], Stéphane Palfi[5]

[1]Sio Gene Therapies Inc., New York, NY,[2]Rho Inc., Durham, NC,[3]Department of Clinical and Movement Neurosciences, UCL Institute of Neurology, London,

United Kingdom,[4]John van Geest Centre for Brain Repair, Department of Clinical
Neuroscience, Addenbrooke's Hospital, Cambridge, United Kingdom,[5]AP-HP, Groupe Hospitalier Henri-Mondor, DMU CARE, Neurochirurgie, Créteil, France

AXO-Lenti-PD (ALPD, previously known as OXB-102), a novel gene therapy for Parkinson's disease (PD), uses a lentiviral vector to deliver the three genes required for endogenous dopamine synthesis (TH, CH1 and AADC), into the putamen with the goal of improving motor fluctuations and long term quality of life. In the Phase I/II study OXB102-01 (SUNRISE-PD; NCT03720418), six subjects were dosed with ALPD: Cohort1 (n=2; Low dose, 4.2E+6 Transducing Units [TU]) and Cohort2 (n=4; Mid dose, 1.4E+7 TU). Efficacy data reported here are from Cohort1 and Cohort2 6-month visits (6M). Given the small sample size, only descriptive statistics were planned. Two of four subjects did not have UPDRS "OFF" 6M data recorded: one due to COVID-19 site limitations and the other declined the OFF assessment. The two evaluable subjects in Cohort 2 had 20.5-point (40%) improvement from baseline (BL) in UPDRS III (Motor Examination) 'OFF'. UPDRS Part II (Activities of Daily Living) "OFF" had 13.5-point improvement (71%). Subjects used the Hauser home diary for up to 3 consecutive days within the week prior to the study visit to log their motor function (either Asleep, ON without dyskinesia, ON with nontroublesome dyskinesia, ON with troublesome dyskinesia, or OFF) at 30 minute intervals normalized to 16 waking hours. Good ON time (the sum of ON without dyskinesia and ON with non-troublesome dyskinesia) increased from BL by 2.2 hours (h) across the four Cohort2 subjects at 6M, while OFF time decreased by 2.3h. Levodopa equivalent daily dose (LEDD) decreased 271.0 mg, 13% lower than at BL. Table 1 summarizes individual and mean changes.

Table 1 Change from Baseline

Subject	OFF Time (h)	Good ON Time (h)	LEDD (mg)	UPDRS II OFF	UPDRS III OFF
Cohort 1					
1001	+2.8	-2.4	-98	-26	-20
1003	-0.5	+2	-150	-13	-14
Mean	**+1.2**	**-0.2**	**-124 (11%)**	**-19.5 (65%)**	**-17.0 (29%)**
Cohort 2					
2002	-0.6	+0.6	0	-12	-22
2004	-0.2	+0.1	-700	-	-
2006	-3.9	+3.5	-466	-	-
2007	-4.6	+4.5	+81	-15	-19
Mean	**-2.3**	**+2.2**	**-271 (13%)**	**-13.5 (71%)**	**-20.5 (40%)**

A total of four serious adverse events (SAEs), all unrelated to ALPD and resolved, have been reported: Parkinson's disease (ie, worsening of non-motor OFF related to anxiety) and Major depression in a Cohort1 subject; Confusional state and Wound infection in a Cohort2 subject. No hypersensitivity, immune or endotoxicity related adverse events have been reported. No serious unexpected suspected adverse reactions (SUSARs) have been reported. No subject died or discontinued from the study. The 6M data from the first two cohorts in SUNRISE-PD trial showed that ALPD gene therapy was generally well tolerated, and the initial efficacy data suggest the potential for a clinically relevant effect. Further evaluation of ALPD is planned using a higher dose/volume open label cohort followed by sham-controlled study.

164. Safety Evaluation of IV-Administered BBP-812, an AAV9-Based Gene Therapy for the Treatment of Canavan Disease, in Mice and Juvenile Cynomolgus Macaques

David W. Scott, Jeremy Rouse, Kirsten Romero, Rachel Eclov, Mayank Kapadia, Daniel McCoy, Clayton W. Beard
Aspa Therapeutics, Raleigh, NC

Canavan Disease (CD) is a rare pediatric leukodystrophy caused by aspartoacylase deficiency. The disease is characterized by elevated levels of the aspartoacylase substrate N-acetylaspartic acid and patients present with a lack of psychomotor development with an average lifespan of less than ten years. We are developing BBP-812, an AAV9-based gene therapy containing a codon modified human *Aspa* transgene, to introduce functional aspartoacylase into CD patients. Proof of concept studies in Aspa -/- mice demonstrated that intravenous (IV) dosing of BBP-812 could reverse signs and symptoms of CD. To better understand the safety of IV delivered BBP-812, we conducted studies in non-human primates (NHP; juvenile cynomolgus macaques) and a GLP-toxicology study in C57Bl/6 mice. The NHP study included IV doses of $3.14x10^{13}$, $1.05x10^{14}$, and $3.14x10^{14}$ vg/ kg with animals sacrificed three and eight weeks after dosing. In the mouse study, animals were dosed at $1.0x10^{14}$, and $3.0x10^{14}$ vg/kg and

were sacrificed four, twelve, and twenty-four weeks after dosing. Safety readouts for both studies included clinical chemistry and hematology, immunology, and microscopic evaluation of major organs systems. For the NHP study, the microscopic evaluation of the nervous system included four spinal cord regions and eight dorsal root ganglia (DRG) per animal. Key findings in the NHP study were a transient increase in ALT and AST in the highest dose IV treatment group at day 3 which returned to normal without intervention by day 8. No other changes in hematology or clinical chemistry occurred during the study. Immunological analysis revealed that all treated animals developed antibodies to AAV9 and the majority of animals developed antibodies to Aspa after dosing. There were no adverse test article-related microscopic changes in the study at any dose level at either necropsy timepoint. Test article-related changes were observed only in the liver (portal infiltrates and/or increased cellularity) in some of the $3.14x10^{14}$ vg/kg treated animals. These findings were considered minor, did not impact the clinical health of the animals, and were not associated with any tissues damage. Evaluation of the spinal cord and DRG revealed sporadic minimal findings that were also present in control animals. Importantly, there

was no evidence of axonopathy in the spinal cord or DRGs as has been reported with other AAV9-based therapies. In the GLP-toxicology study in mice there were no adverse test article related changes in any clinical chemistry or hematology during the study. Creatine kinase and lactate dehydrogenase were decreased at both dose levels at all timepoints but this was not considered adverse. Immunological analysis revealed that all BBP-812 treated animals generated antibodies to AAV9 while no animals generated antibodies against Aspa. T-cells were monitored for reactivity to AAV9 and Aspa. There was a minor response in some animals to AAV9 with no response detected towards Aspa. There were no microscopic changes in the study in any group at any time point. Based on these findings, the NOAEL from the NHP study was determined to be $3.14x10^{14}$ vg/kg and from the GLP toxicology study in mice was determined to be $3.0x10^{14}$ vg/ kg of IV-administered BBP-812. These studies support the continued development of BBP-812 for the treatment of CD.

165. Gene Replacement Therapy for *SURF1*-Related Leigh Syndrome Using AAV9

Qinglan Ling, Matthew Rioux, Steven Gray
UTSW, Dallas, TX

SURF1(surfeit locus protein 1)-related Leigh syndrome (LS) (also known as Charcot Marie Tooth disease type 4K) is an early onset neurodegenerative disorder characterized by reduction in the assembly factor of complex IV, resulting in disrupted mitochondrial function. Here, we hypothesized that functional gene replacement strategy could restore mitochondrial functions in LS caused by *SURF1* loss-offunction mutations. A codon-optimized version of the human *SURF1* (h*SURF1*) packaged within self-complementary adeno-associated virus serotype 9 (AAV9) viral vectors (AAV9/h*SURF1*), which have been shown to induce robust expression in central nervous system (CNS), was designed and generated as the treatment entity. Our and other studies indicated that the knockout (KO) of *SURF1* in mice reduced complex IV/cytochrome *c* oxidase (COX) activity and MT-CO1 (mitochondrially encoded cytochrome *c* oxidase I) protein expression in multiple organs, as well as induced lactic acidosis. Thus, we treated juvenile *SURF1* KO mice with AAV9/h*SURF1* through broad CNS-

directed delivery (intrathecal (IT) administration, or a combination of intrathecal and intravenous administration), in a manner that is compatible with human translation. Our data indicate that 4 weeks after intrathecal administration into juvenile mice, the COX activity was partially and significantly rescued in all tissues tested, including liver, brain and muscle. Furthermore, our histology study suggest AAV9/ h*SURF1*-treated mice showed dose-dependently increased h*SURF1* mRNA expression and restoration of MT-CO1 protein expression in the brain, which further supported our findings in COX activity. Additionally, we tested endurance capacity and lactic acidosis 4 weeks and 9 months after the treatment in a separate group of mice. Our data suggest that the gene replacement treatment also mitigated the lactic acidosis upon exhaustive exercise at mid-age. However, doubling the total amount of virus by administering through both intravenous and intrathecal route did not confer any significant improvement compared to an intrathecal route alone in any of the parameters tested above, except the COX activity in liver. This suggests a single dose IT administration of AAV9/h*SURF1* is effective and sufficient in improving SURF1 deficiency-related dysfunctions. The toxicity of the vectors was evaluated through intrathecal administrating into wildtype (WT) mice at a maximum feasible dose. There were no toxicities observed in either the in-life portion of the study or after microscopic examination of major tissues up to a year following gene transfer. Taken together, we propose gene replacement therapy through IT administration of AAV9/ h*SURF1* as a potential treatment worthy of further development for *SURF1*-related LS patients. Further, this general approach might be amenable as a potential treatment for patients with other related forms of LS or other mitochondrial disorders.

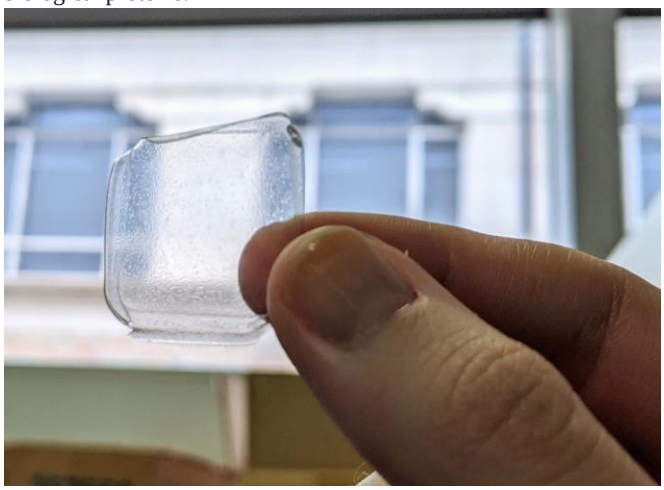
Downstream Process of Vector Manufacturing

166. Novel Platform for Transport and Delivery of Recombinant Adeno-Associated Virus without Need for Cold Storage during Transit

Maria A. Croyle[1,2,3], Trang Doan[1], Matthew D. Le[1], Irnela Bajrovic[1,4], Lorne Celentano[4], Charles Krause[4], Haley G. Balyan[4], Abbie Svancarek[4], Lakmini Wasala[4], Angela Mote[4], Anna Tretiakova[4], R. Jude Samulski[3,4]
[1]College of Pharmacy, University of Texas at Austin, Austin, TX,[2]John R. LaMontagne Center for Infectious Disease, Austin, TX,[3]Jurata Thin Film, Chapel Hill, NC,[4]AskBio, Research Triangle Park, NC

Within the past 30 years, significant progress has been made in the development of gene vectors with several therapies receiving approval by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA). Despite this progress, these products are stored and shipped at $\leq$ -70°C, similar to current COVID19 vaccines. This poses significant logistic and economic issues with respect to global distribution and access to life-saving medicines. In an effort to address these important issues, we developed a novel method for stabilizing live viruses in a peelable thin film matrix that can be stored at ambient temperature (see figure). In these studies, an AAV9 vector (AAV9-CBALuc) was mixed with film base formulation, poured into 1 ml molds and dried under aseptic conditions. Films were peeled and packaged in individual particle free-bags with foil overlays and stored at room temperature under controlled humidity. A sublot of films were shipped from Texas to North Carolina via overnight courier in an envelope without ice or cold blocks for in vivo testing. Control preparations were stored in a commercial liquid formulation at room temperature and 4 and -80 °C for comparison. Over 95% of the original infectious dose of AAV was recovered after drying as determined by *in vitro* transduction assays measuring transgene expression and internalized viral genome copies. In contrast, infectious virus particles could not be detected in the liquid formulation after 30 days at room temperature with large aggregates detected by dynamic light scattering at 14 days transitioning to

subviral particulates at day 30. Stability profiles of the same preparation stored at 4 °C for 30 days were not statistically different from vector stored in film at room temperature at the same time point. Films taken from room temperature storage and placed at 40 °C with 20% relative humidity for 3 days demonstrated a loss of less than 5% of infectious titer. Initial mechanistic studies suggest that elements of the film matrix directly bind to capsid proteins to stabilize them and shield them against environmental stressors. *In vivo* studies in mice demonstrated that vector biodistribution and transgene expression profiles of vector dried within the film matrix were similar to those of vector stored frozen in the commerical formulation. Taken together, these results suggest that storage of AAV in our novel matrix facilitates easy transport of vector to remote sites without compromising in vivo performance. Companion studies using enveloped and non-enveloped viruses (Ad, influenza) further suggest that this approach could be a universal technology for room temperature storage of vaccines, gene therapy vectors, and biological proteins.



167. Exposing the Content of Different AAV Fractions after Ultracentrifugation

David Dobnik, Mojca Janc, Maja Štalekar, Nejc Košir, Tjaša Jakomin, Rebecca Vollmeier Kovačić, Nika Savodnik, Polona Kogovšek

National Institute of Biology, Ljubljana, Slovenia

Recent developments in the biomedical fields have enabled that viruses can also be used to help us fight against numerous diseases. Even though the production of recombinant viral vectors began 40 year ago, the established technologies still haven't reached the optimal level.

Different product and process related impurities affect the efficiency of the production. To improve the overall production process, advanced analytical techniques are needed to accurately follow presence of desired product and possible impurities in various production steps. A variety of methods have been developed and utilised to characterize viral vectors, but only a limited number of comparative studies have been done to demonstrate correlations between them. Non-related analytical techniques usually also produce non-comparable results. We have used different analytical techniques (Quantitative realtime PCR (qPCR), digital droplet PCR (ddPCR), high throughput sequencing (HTS), enzyme-linked immunosorbent assay (ELISA) and transmission electron microscopy (TEM)) to thoroughly characterize four fractions of AAVs (empty, intermediate, full and heavy particles) coming from CsCl gradient purification. The aim of the study was to compare those different fractions in terms of vector and impurities content and at the same time evaluate the correlations between different analytical methods. The results have shown that the fractions were relatively similar in terms of relative content of vectors and impurities, with exception of heavy fraction. There was no clear correlation between the results of different methods that would hold for all of the fractions. Nevertheless, the results offered an interesting insight into the presence of impurities, vector genomes and viral particles in different fractions. Those results will be presented and discussed in view of content of partially filled vectors, comparability of analytical methods and importance for creating a wider picture of the virus vector sample.

168. Optimization of Affinity Purification for Adeno-Associated Viral Vectors

Huiren Zhao, Songli Wang, W. Hans Meisen

Genome Analysis Unit, Amgen Inc., South San Francisco, CA

Purification of recombinant AAV (rAAV) via affinity chromatography with Poros™ CaptureSelect™ (PCS) AAVX, AAV8 or AAV9 affinity resins achieves good purity and high vector concentration with minimal vector loss. Additionally, this purification process is highly scalable. Despite the advantages of affinity purification, we and others have observed that significant host cell proteins (HCPs) and other impurities such as endotoxins are co-eluted with rAAV. The goal of this study was to optimize the washing step during affinity chromatography to remove contaminants and increase rAAV purity. In this study, we systematically evaluated a variety of washing reagents including 1M NaCl, 0.2 M $MgCl_2$, low pH (pH4 and pH5), 0.5 M arginine, 0.1% Tween 20 and 1-2% OTG (octyl β-D-1-thioglucopyranoside) for their ability to improve rAAV8-EGFP affinity purification using PCS AAVX. In these experiments purity was evaluated by Coomassie blue stain of SDS-PAGE gels, titer by the CyQuant method, and endotoxin by the

Limulus Amebocyte Lysate (LAL) method. A large batch of rAAV8EGFP was generated and used as the starting material for all rAAV8 affinity purifications. Gradients of wash buffers were applied in some purifications to identify the best concentration of the reagent. In these studies, we observed that some of the wash buffers tested resulted in the loss of rAAV8 by capsid protein immunoblot including 0.1% Tween 20, 50 mM Citrate buffer (pH 4.0) and 0.5 M arginine. These findings highlight the challenge of identifying washing buffers that can remove impurities while retaining the association of the rAAV and the affinity ligand. rAAV8 vector was not washed off by 1M NaCl, 0.2 M $MgCl_2$, 50 mM Citrate buffer (pH 5.0), or 2% OTG as determined by immunoblot.

Compared to PBS wash alone, these reagents reduced the amount of contaminating proteins and endotoxins (about 66-100%) from the vector preparation. Among the reagents evaluated, 2% OTG resulted in the greatest reduction in contaminating proteins and endotoxins (<LoD) compared to PBS wash alone. Using this protocol we achieved yields approximately 88% of PBS wash alone suggesting OTG wash results in minimal vector loss. Our preliminary data also showed that OTG wash can be used to purify rAAV1 (1% OTG wash), rAAV2 (1% OTG wash), and rAAV5 (2% OTG wash) with PCS AAVX resin. A 2% OTG can also be used to successfully purify rAAV9 with PCS AAV9 resin. We observed approximately half of rAAV9 was lost after 2% OTG wash using PCS AAVX resin suggesting differences in how rAAV9 interacts with the two resins. Currently, other serotypes are being tested with OTG wash. Our group previously published an optimized rAAV production protocol in suspension HEK 293T cells with tripletransfection (Zhao, et al. Mol Ther Clin Dev & Methods, 2020). When this OTG wash was combined with a modified protocol that eliminates sodium butyrate, we were able to produce about 5-6×10^{14} VG of purified rAAV8-Ef1α-EGFP from one liter of suspension HEK 293T cell culture. The yield achieved via this suspension HEK 293T cell platform and our affinity purification scheme offers high scalability and a comparable yield to *sf9* cells (Kurasawa et al. Mol Ther Clin Dev & Methods, 2020) making it an appealing option for rAAV generation.

169. Characterization of rAAV Key Quality Attributes Generated from a Highly Optimized, Hela 3.0 Producer Cell Line (PCL) Production Platform

Nicholas Richards[1], Caroline Adams[2], Kevin Larpenteur[2], Justin Tedeschi[2], Samuel Wadsworth[2], K. Reed Clark[2], Matthew Fuller[2]

[1]Ultragenyx Pharmaceuticals, Inc, Cambridge, MA,[2]Ultragenyx Gene Therapy, Cambridge, MA

A key challenge facing the gene therapy field today is ensuring that manufacturing capabilities surpass current standards to ensure accessibility and affordability for all eligible patients, as well as facilitating treatments for diseases that require higher doses for efficacy, such as Duchenne muscular dystrophy. To address these needs, we have developed a HeLa-based rAAV manufacturing platform that has demonstrated scalable 2000L production in an industrial setting to support ongoing Phase I/II clinical trials. Recently, we have further optimized our HeLa Producer Cell Line (PCL) platform to HeLa 3.0 by genetically modifying existing, highly productive monoclonal PCLs. Utilizing an RNA-seq directed screening method, we identified specific genes that modulate rAAV production and knocked out those genes *via* CRISPR/Cas9-mediated genome editing, which increased productivity up to 5 fold. While titer improvements are important, ensuring the quality and fidelity of both the production platform and manufactured vector is vital. To this end, two HeLa 3.0 producer cell lines, developed for separate clinical programs, were interrogated to characterize attributes previously identified as essential for generation of a robust, stable cell line including rAAV genome amplification, integration site analysis, kinetic analysis of rAAV production and overall fitness. In addition, viral products generated from both HeLa 3.0 PCLs were analyzed for key quality attributes, such as genome integrity,

capsid protein ratio, and empty: full ratio to establish comparability with existing, efficacious vectors. Importantly, the newly optimized HeLa 3.0 production system demonstrated the robustness required to facilitate production of high quality rAAV vector product.

170. AAVX Resin Binding Site Identification via Library Screening Analysis on Novel AAV Vectors

Stewart Craig[1], Zachary Thorpe[1], Rebecca McDonnell[1], Kimberly Le[1], Allegra Fieldsend[1], Deepak Grover[1], Sri Siripurapu[1], Stephanie Malyszka[1], Laura K. Richman[1], Luk H. Vandenberghe[1,2], Christopher Tipper[1]

[1]Affinia Therapeutics, Waltham, MA,[2]Grousbeck Gene Therapy Center, Mass Eye and Ear and Harvard Medical School, Boston, MA

A major effort by AAV-based gene therapy manufacturers is focused on improving yield and reducing COGs independent of AAV serotype to enable the potential treatment of rare and non-rare diseases with a one-time dose of gene therapy. Key among these efforts is improved affinity capture. Affinity capture is a scalable method that effectively removes host impurities from AAV vector preparations. As with any receptor/ligand complex, the stringency of capture of the AAV particle is a function of the dissociation constant (Kd) of the interaction. Identifying and understanding the specific chemistries of the interaction may allow engineering and modulation of the Kd. Similar to AVB, AAVX is an affinity resin that utilizes a camelid antibody to capture AAV vector particles. Camelid antibodies are single chain and exhibit extended CDRs, making them ideal for reaching confined epitopes and conjugation to porous substrates. The development of the resin by ThermoFisher has been recognized as a major improvement to downstream processes. AAVX resin can bind most tested serotypes with an affinity high enough to withstand the stringent wash conditions required for therapeutic development. Here, we investigate the potential AAV epitope responsible for binding to AAVX resin. The use of AAV vector libraries to address manufacturability concerns has not been widely reported. The rich datasets produced using diverse yet informatically manageable vector libraries engineered via ancestral sequence reconstruction provide an opportunity to answer questions of manufacturability at multiple process steps. Exemplifying this utility, we have preliminarily identified the region of the AAV vector where the AAVX epitope resides. Processing the Anc80 vector library via AVB and AAVX affinity resins revealed differential efficiency of vector capture. By informatic analysis, the single residue responsible for this observation was identified. Interestingly, this binding site is located within the 3-fold axis, which is distinct from the AVB binding site near the 5-fold axis. Further studies to

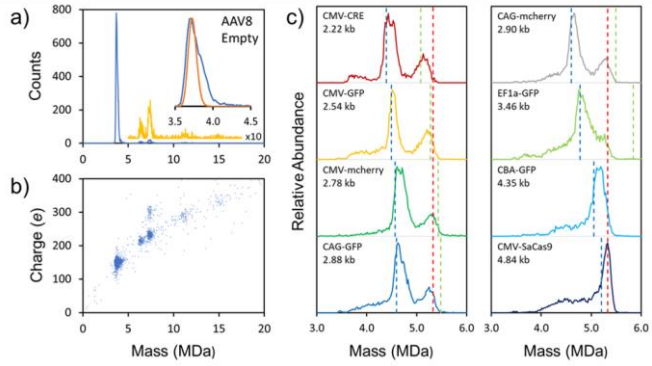
[1] Megadalton Solutions, Bloomington, IN,[2]Chemistry, Indiana University, Bloomington, IN

definitively identify critical primaryto-tertiary elements of the AAVX epitope are ongoing.

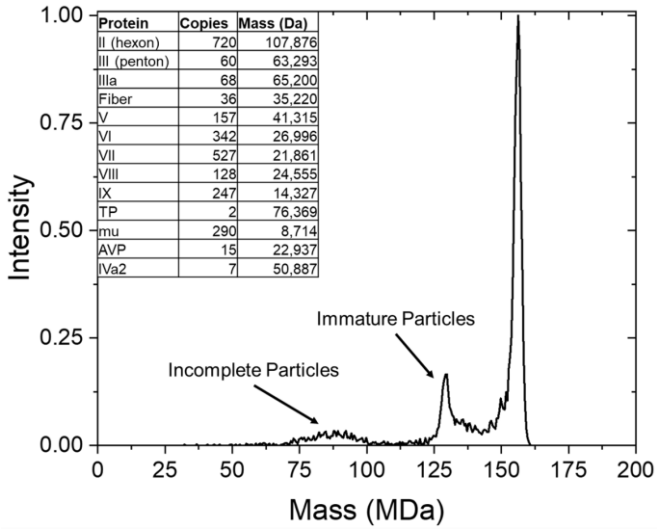
171. Analysis of Gene Therapy Products by Charge Detection Mass Spectrometry

Benjamin E. Draper[1], Lauren F. Barnes[2], Martin F. Jarrold[1,2]

New analytical tools in cell and gene therapy will improve patient outcomes. In general, commercial mass spectrometers have an effective upper mass limit around a megadalton (MDa), a limit that arises because of the loss of charge state resolution in the mass-tocharge (*m*/*z*) spectrum. Cell and gene therapy platforms fall beyond this range, but a relatively new technique called charge detection mass spectrometry (CDMS) can circumvent this limitation and bring the precision of mass spectrometry to cell and gene therapy. CDMS simultaneously measures the *m/z* and charge (*z)* of individual particles allowing for a direct calculation of mass. This extends accurate mass measurements into the MDa to gigadalton (GDa) regime. Accurate mass measurements obtained from CDMS can be used to assess a range of critical quality attributes for gene therapy products. This work will highlight how CDMS has been demonstrated for viral vector platforms and genomic material. Adeno-associated virus (AAV) vectors have a mass in the 3-5 MDa range depending on the packaging of genomic material. Direct mass measurement allows for the packaging to be assessed and the gene of interest to be identified for intact capsids. We show that the differences between the masses of empty particles and particles with the genome of interest (GOI) are correlated with the expected genome mass. In addition, CDMS provides a robust orthogonal technique to quantify empty, partial, and full particles as well as aggregates that form through both capsid degradation and capsid adherence. Adenovirus vectors have a well-established use in cancer treatments and hold great promise in vaccine research. Here we show the first high resolution mass measurement for a virus with a mass over 100 MDa. Using CDMS, we correlate the measured mass for a range of packaged genome lengths to the amount of co-packaged counterions and proteins important for genome stability. In addition, we use the accurate charge measurement to assess structure and identify incomplete, defective, and empty particles of adenovirus. Due to the importance of genomic material in gene therapies, we show how CDMS can assess the purity and mass of plasmids. Building off the mass measurements of plasmids we also able to directly detect and measure the genomic material from disassembled AAV and adenovirus capsids. In the case of AAV both ssDNA and dsDNA corresponding to the GOI were detected and used to identify the purity of the packaged contents. All spectra and results showed above use a single CDMS platform that is robust and highly sensitive. A few benchmarks include: AAV analysis low titer samples (1e10 vp/mL) in heterogeneous matrices, mass measurements with low volumes (10-20 μL), and analysis times of less than 1 hour.



(Figure 1)



(Figure 2)

172. Use of SPTFF in Continuous Downstream Manufacturing of Adeno-Associate Viruses

Rajeshwar Chinnawar, Shawn Tansey, Nicholas Marchand

R&D, Pall Biotech, Westborough, MA

In recent years, pre-clinical and clinical development in the gene therapy industry has been rapidly growing. To meet the industry's requirement for large quantities of GMP-compliant therapeutic viral vector, there's a need for high-efficiency equipment and consumables. A typical downstream process for AAV manufacturing involves a combination of unit operations including clarification of crude harvest, chromatography, concentration, diafiltration and sterile filtration. A few challenges in AAV processing include processingtime and shear sensitivity of the product, and safety concerns of the product. Adding TFF membranes into a process can reduce working volumes, and application of single-use consumables can mitigate safety concerns. Replacing traditional recirculating TFF with newer Single-Pass TFF (SPTFF) technologies has the potential to reduce shear exposure, reduce processing time by integrating with unit operations before and/or after, and improve process yields. In this work we implemented Palls innovative Cadence SPTFF technology for in-line concentration to optimize an AAV downstream process. An SPTFF device was connected to an upstream depth filtration assembly with a small break tank. The post-

SPTFF-concentrated viral vector stream was continuously pumped through a sterile filter. This work demonstrates the use of an integrated, continuous SPTFF operation in an AAV process that achieves 40% reduction in processing time while maintaining a 96% Yield.

Immunotherapy and Vaccines

Immunotherapy and Vaccines

173. A Humanized EBV Mouse Model to Evaluate the Safety/Impact of Human T_{reg} Cell Therapy on Antiviral Immune Responses

Swati Singh, Stefan Lachkar, Noelle Dahl, Christina Lopez, Yuchi Honaker, Claire Stoffers, Anna ZielinskaKwiatkowska, Iram Khan, Karen Sommer, David J. Rawlings
Seattle Children's Research Institute, Seattle, WA

Regulatory T cells (Treg) suppress immune cell activation and thus have key roles in preventing excessive immune responses and autoimmunity. Adoptive cell therapy with Treg is currently being tested clinically to provide tolerance for graft vs. host disease, organ transplant and autoimmune diseases. Due to potential limitations of Treg frequency, stability and ex vivo expansion, we have utilized gene editing to enforce FOXP3 expression in peripheral blood $CD4^+$ cells, driving them to adopt Treg-like phenotypes and functions. We refer to these cells as engineered Tregs (Eng Tregs). While Eng Tregs show robust suppression of pathological immune responses in mouse models, it is theoretically possible that adoptively transferred Treg could have undesirable off-target effects such as suppressing immune responses against pathogens, either during novel exposures or the reactivation of latent viruses such as Epstein-Barr virus (EBV). While most humans (95%) will have been infected with EBV by adulthood, infection is usually well-controlled by the immune system. However, a reservoir of latently infected B cells persists for decades, kept in check by immunological surveillance; the latent EBV can become reactivated when the immune system is suppressed. The goal of our study was to evaluate the safety/impact of engineered Treg (Eng Tregs) on viral responses. Since EBV does not infect mouse cells, we tested this in the following humanized mouse model. From a G-CSF-mobilized human donor, we purified autologous $CD34^+$ peripheral blood stem cells (PBSC), $CD4^+$ T cells, and thymic Treg (tTreg). The $CD34^+$ PBSCs were transplanted into busulfan-conditioned NOD-*scid*-$IL2Rg^{NULL}$ (NSG) mice. After stable engraftment (12-13-weeks post-transplant), humanized NSG were infected with EBV, then subsequently treated with either autologous tTreg or Eng Tregs ($CD4^+$ T cells edited to stably express FOXP3 utilizing HDR-based CRISPR/Cas9 targeted integration of FOXP3 cDNA with an MND promoter into *FOXP3)*. Eight weeks later, we quantified circulating EBV loads and the immunophenotype of human lymphocytes in the spleen, blood, and bone marrow. These experiments included control mice that were treated with T celldepleting antibodies in place of Treg; these mice rapidly developed B cell proliferative disease associated with EBV infection/re-activation and had high EBV loads vs. mice receiving EBV alone. This control demonstrated the role of the human $CD4^+$ and $CD8^+$ T cells in keeping the EBV infection in check in this model. Importantly, the viral loads, % human B cell and % activated human $CD8^+$ T cells in response to EBV infection were equivalent between mice that received Eng Tregs, tTreg, or EBV alone. Further, we were able to detect $LNGFR^+$ $FOXP3^+$ cells in the Eng Tregs cohort at his time point, suggesting the stability of Eng Tregs in this pro-inflammatory setting. These combined data demonstrate that Eng Tregs do not impact the immune responses required to control EBV infection in this EBV-infected humanized mouse model; demonstrating an effective in vivo system to evaluate whether human Treg-based cell therapies can preserve desired antiviral immune responses.

Immunotherapy and Vaccines

174. Candidate Selection in BALB/c Mice towards a Single Dose AAV-Based COVID19 Prophylactic Vaccine

Wenlong Dai, Nerea Zabaleta, Urja Bhatt, Reynette Estelien, Dan Li, Dawid Maciorowski, Julio Sanmiguel,
Ruchi Chauhan, Allison Cucalon, Cheikh Diop, Maya Kim, Abigail Sheridan, Luk H. Vandenberghe
Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

The public health crisis of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARSCoV-2) exceeds 100 million cases worldwide. While several protective vaccines are becoming available, several challenges to attain levels of population immunity remain. We hypothesized that, based on prior developments, an AAV-based preventative vaccine could address some of the biological and logistical limitations to an effective vaccination campaign at a global scale, importantly for example single dose efficacy. In the present study, we designed 9 AAV2/rh32.33 vector based COVID-19 genetic vaccine candidates (AAVCOVID) expressing the wild type full length spike (S) protein, stabilized S derivatives and several soluble S-based antigen, namely S ectodomain, S1 and receptor binding domain (RBD), the latter both in ssAAV and scAAV context. Wuhan S and D614G variant strains were used as the basis for antigen design. AAVCOVID candidates were evaluated for immunogenicity following single dose intramuscular (IM) injection in BALB/c mice at either 10^{10} gc/mouse or 10^{11} gc/ mouse for a period of 3 months post injection. From these studies, a candidate selection was performed for further development. Results demonstrate variable levels, yet overall rapid and robust antigen specific binding and neutralizing antibody responses. Interestingly, serum antibody isotype profiles differed qualitatively between membrane anchored antigens and soluble antigens, suggesting a more pronounce Th2 phenotype for the soluble antigen vaccine candidates. However, all candidates induced robust IFN-γ, but not IL4, T cell response by ELISpot assay at a 3-month time point, indicative of a Th1 biased response for all AAVCOVID candidates. For the duration of the immune response, the serum antibody titer reached the peak at month 2 and started to decrease at month 3. Remarkably, T cell responses in the low dose group mice were equivalent to modestly higher than those in high

dose group. Taken together, these results indicate that the AAVCOVID platform induces high level neutralizing responses that are complemented with robust T-cell immunogenicity. Based on these data and other strategic parameters, AAVCOVID-1 (membrane anchored pre-fusion stable S) and AAVCOVID-3 (secreted S1) progressed to further studies toward consideration of one candidate for clinical development.

175. AAV Specific CAR Regulatory T Cells Mitigate Immune Responses against AAV Gene Therapy

Motahareh Arjomandnejad[1], Thomas Nixon[1], Qiushi Tang[1], Meghan Blackwood[1], Katelyn Sylvia[1], Alisha M. Gruntman[1,2,3], Guangping Gao[1], Terrence Flotte[1,2], Allison M. Keeler[1,2]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA,[3]Department of Clinical Sciences, Department of Clinical Sciences, Cummings School of Veterinary Medicine at Tufts University, Grafton, MA

Despite the success of AAV vectors as powerful gene therapy tools, the immune responses to AAV capsids have limited their therapeutic applications. As observed in clinical trials, systemic delivery of AAV leads to capsid specific immune responses and clearance of the transgene by CD8 T cells while intramuscular delivery of AAV, leads to long-term transgene expression despite an immune response. Differences in transgene expression is thought to be due to T regulatory cell (Treg) infiltration in muscles creating tolerance to AAV capsids. Hence, we examined the ability of Tregs to induce local and specific immunosuppression rather than steroid mediated non-specific immune suppression. We previously generated AAV specific chimeric antigen receptor T cells (AAV-CAR), and AAV specific regulatory T cells (CAR-Treg) which recognize major AAV capsid variants. AAVCARs are cytotoxic to AAV infected target cells and this killing is suppressed by AAV-CAR-Tregs. In murine models, AAV-CARs cleared AAV infected cells. Herein, we confirmed the cytotoxicity of AAVCARs and the suppressive ability of AAV-CAR-Tregs against the AAV capsid variants- AAV1, AAV2, AAV3b, AAV 5, AAV6, AAV8, AAV9 and rh32.33. Further, AAV-CAR-Tregs inhibited the proliferation and interferon gamma (IFN-γ) production of AAV-CAR T cells when stimulated with AAV capsid. Next, we utilized AAV-rh32.33 capsid to elicit a robust immune response in mice. Animals were injected with AAV-rh32.33 expressing alpha 1 anti-trypsin (AAT) followed by intravenous delivery of AAV-CAR-Tregs, non-specific expanded T-regs, or saline. AAT expression was stable for 25 weeks in both Treg and AAV-CAR-Treg groups, despite high level of anti-capsid antibody with no detectable expression 3 weeks post AAV injection in the saline group. Histology of injected muscles revealed detectable AAT staining in AAV-CAR-Treg, or non-specific Tregs groups and not in the untreated group. Tregs can induce tolerance in an antigen independent pathway via the bystander effect, where antigen activated Tregs create local suppressive environment which can suppress other antigens. To determine if AAV-CAR-Tregs can function similar to endogenous Tregs, we tested if they AAV capsid specific AAV-CAR-Tregs could block immune responses to transgene. We designed a cytotoxicity assay with mixed antigens of CAR T cells and CAR-Tregs which revealed that AAV-CAR-Tregs could suppress CAR T cells specific to other antigens. To test this principle in vivo, we delivered AAV1 expressing the immunogenic OVA (AAV-OVA) transgene followed by delivery of either AAV-CAR-Tregs, expanded non-specific T-regs, or saline. Stable transgene expression was observed in the animals receiving AAV-CAR-Tregs, or non-specific T-regs, and was reduced 2 weeks after AAV-OVA in the saline group. Interestingly, all groups produced anti-OVA antibodies despite treatment. Moreover, histology of injected muscles revealed severe focal, and diffuse myositis in untreated animals, with significantly reduced inflammation observed in the muscles of the animals treated with AAV-CAR-Tregs, despite a robust OVA expression. Together, this data demonstrates that AAV-CAR-Tregs are powerful tools to model the cellular immune response against AAV capsid. Therapeutically, AAV-CAR-Tregs potentially can modulate immune responses directed against AAV capsids and immunogenic transgenes without the detriments of systemic immunosuppression.

176. Engineered Protein M Analogs Enhance the Ability to Suppress Vector Neutralizing Antibodies and Generate a Window for Successful Gene Delivery

Charles Askew, David Thieker, Zheng Chai, Brian Kuhlman, Chengwen Li

University of North Carolina, Chapel Hill, NC

Patient derived neutralizing antibodies (NAbs) to viral vectors are a major hurdle to using these advanced biologics as traditional drugs. NAbs block therapeutic gene delivery in those with pre-existing vector immunity, and prevent vector dose titration after the first treatment. Of the strategies in development to reduce the effect of pre-existing NAbs or blunt the generation of antibodies during treatment, most suffer from limited efficacy to bring NAb titers below a level that permits vector administration, and none demonstrate instantaneous NAb suppression. Previously we demonstrated that a soluble fragment of mycoplasma Protein M transiently blocks NAbs to adeno-associated virus (AAV), which enabled successful in vivo systemic gene delivery over a 1,000-fold concentration range of antiAAV NAbs as compared to controls without Protein M where the same AAV dose was neutralized. Protein M binds to the Fab region of all antibody classes at a conserved site common to humans, non-human primates, rodents, and most mammalian species. Protein M binding temporarily blocks all antigen recognition through steric hinderance of the complementarity-determining regions, and generates a transient gene delivery window whereby any vector is universally protected from neutralization. Additionally, we demonstrate that use of Protein M with a variety of vectors instantaneously blocks NAbs for successful gene delivery, enabling robust gene expression in the presence of neutralizing serum. While generating feasibility data we quickly discovered that Protein M is highly unstable at body temperature, posing a significant challenge for in vivo use and scalable protein manufacturing. Instantaneous Protein M unfolding occurs at 41°C, resulting in precipitation and aggregation. Incubating a truncated naturally occurring Protein M sequence at 37°C caused protein

unfolding after only 15 minutes and loss of antibody blocking function. Therefore, we used structural and computational modeling to design 850+ Protein M analogs with enhanced properties. We produced a library of stabilized mutants using *in silico* site saturation mutagenesis and free energy prediction, and then screened single and combinatorial mutants. Stabilizing mutations resulted in a distribution of analogs with increased melting temperatures, from 42°C degrees to 65°C and greater. Improvements in thermostability were accompanied by significantly decreased aggregation, increased solubility, and ability to highly concentrate the protein. Stabilized Protein M variants improved in vitro function, whereby several analogs retained antibody blocking

ability after more than 72hrs at 37°C. Using an engineered analog, PM129, we now demonstrate successful readministration of AAV in mice immunized by AAV one-month before secondary administration. PM129 blocks NAbs after systemic intravenous dosing shortly before AAV administration, as well as when PM129 is admixed with AAV and administered in a single intramuscular injection. While PM129 is a novel foreign protein with the capacity to elicit inhibitory antibodies to itself, we are able to successfully redose PM129 and AAV even when prior doses of PM129 have been administered. This suggests PM129 is capable of outcompeting self-neutralizing antibodies, which likely represent only a small fraction of the total serum antibody pool. Finally, safety assessments of PM129 were conducted in mice demonstrating no observed adverse reactions in over 40 mice included in the high dose cohort. Minimal B cell or T cell proliferation was observed after in vivo administration and collected serum samples failed to activate complement C5 or demonstrate immune complex formation.

177. *In Vivo* HSC Gene Therapy with High-Level, Erythroid-Specific Expression of a Secreted SARS-CoV-2 Decoy Receptor

Hongjie Wang[1], Sucheol Gil[1], Chang Li[1], Christopher Doering[2], Philip Ng[3], Steve Roffler[4], Hans-Peter Kiem[5], Andre L. Lieber[1]

[1]University of Washington, University of Washington, Seattle, WA,[2]Emory University, Atlanta, GA,[3]Baylor College of Medicine, Houston, TX,[4]Academia Sinica, Taipei, Taiwan,[5]FHCRC, Seattle, WA

We developed a technically simple and portable *in vivo* hematopoietic stem cell (HSC) transduction approach that involves HSC mobilization from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAd5/35++ vectors target human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive Sleeping Beauty transposase (SB100x) and transgene marking in peripheral blood cells can be increased by *in vivo* selection. Here we directed transgene expression to HSC-derived erythroid cells using beta-globin regulatory elements. We hypothesized that the abundance and systemic distribution of erythroid cells can be harnessed for high-level production of secreted therapeutic proteins. We first demonstrated that our approach allowed for sustained, erythroid-lineage expression of a bioengineered human factor VIII, termed ET3, leading to phenotypic correction of the bleeding defect in $hCD46^{+/+}/F8^{-/-}$ hemophilia A mice as measured by chromogenic assay, aPTT assays and tail bleeding assays (PMID: 31585952). We then used this approach for continuous expression of a SARS-CoV-2 decoy receptor, i.e. a secreted, human ACE2 extracellular domain fused to human constant IgG1 domains to form an antibody-like structure (sACE2-Ig) capable of mediating virus opsonization. We performed *in vivo* HSC transduction of CD46-transgenic mice with a HDAd5/35++-sACE2-Ig vector. Serum sACE2-Ig levels reached 500-1300 ng/ml after *in vivo* selection. The sACE2-Ig protein in the serum from these animals was active in blocking the infection of $293\text{-}ACE2^{+}$ cells with a CoV2-Spike proteinpseudotyped lentivirus vector. Lineage-negative bone marrow cells from HDAd5/35++-sACE2-Ig-transduced CD46-transgenic mice will be transplanted into lethally irradiated K18-hACE2 transgenic

mice. The resulting mice will express sACE2-Ig and will be susceptible to SARS-CoV-2 infection. Furthermore, we performed *in vivo* HSC transduction with the integrating HDAd5/35++sACE2-Ig vector in a rhesus macaque. HSC mobilization by subcutaneous administration of G-CSF/plerixafor and intravenous HDAd injection at dose of $3.2x10^{12}$ vp/kg was well tolerated after appropriate cytokine prophylaxis (dexamethasone, tocilizumab, anakinra). The HSC transduction efficacy and serum sACE2-Ig levels after *in vivo* selection will be reported. Ultimately, we plan to test whether continuous sACE2-Ig expression could provide protection against challenges with CoV-2 variants in the mouse and NHP models.

178. A Platform for Genome Editing of Human B Cells to Produce Single-Chain Antibody-Like Molecules That Recapitulate Antibody Functionality

Geoffrey L. Rogers, Xiaoli Huang, Chun Huang, Atishay Mathur, Paula M. Cannon

Molecular Microbiology and Immunology, University of Southern California, Los

Angeles, CA

Genome editing of B cells offers opportunities to create cells that express antibodies with characteristics that cannot be elicited by vaccination. However, the complex regulatory pathways governing antibody function, which include alternative splicing, class switch recombination, and somatic hypermutation, suggest that editing the endogenous immunoglobulin (Ig) locus may be required to fully reconstitute such functions in an engineered antibody. Towards that goal we have developed a strategy to engineer the human Ig locus based on the design of single-domain antibodies (sdAbs) - heavy-chain only antibodies found in camelids that comprise an antigen-binding VHH domain and heavy chain Fc region. By inserting a promoterVHH-splice donor cassette within an intron of the human IgG1 gene (*IGHG1*), a spliced mRNA is produced that fuses the VHH to constant domains of IgG1 and results in both a recombinant membrane-bound B cell receptor (BCR) and the matched secreted

antibody. As a proof of principle, we used CRISPR/Cas9 to engineer human B cell lines with HIV Env-specific VHH domains. We observed anti-HIV BCR cell surface expression, signaling after engagement with the HIV Env antigen, and secretion of functional sdAbs capable of neutralizing HIV. Long-term culture of the cells resulted in signatures of somatic hypermutation, with the sequence changes strongly localized to AID hotspot motifs, and consequential alterations in antibody functionality. Such an ability may provide advantages against a highly mutable target such as HIV. Expanding the approach to primary human B cells, we combined CRISPR/Cas9 with AAV6 homology donors to insert anti-HIV VHH cassettes at *IGHG1*. Over 20% of cells expressed HIVspecific human IgG as measured by flow cytometry, and the engineered B cells could be expanded >50-fold over 11 days using an animalcomponent free system. After *in vitro* differentiation that skewed the B cells towards an antibody-secreting cell phenotype, we observed a shift in splicing of the chimeric antibody mRNA towards the secreted isoform and increased anti-HIV sdAb secretion. Importantly, antiHIV sdAb secretion tracked with total IgG secretion by unmodified cells on a per cell basis (R^2=0.99), indicating that alternative splicing of the transgene was co-regulated with endogenous antibody splicing by the differentiation state of the B cell. Finally, supernatants from engineered B cells were able to neutralize HIV infection with an IC50 equivalent to the recombinant VHH-Fc protein, indicating that sdAbs generated by this strategy are properly produced and secreted despite the presence of other endogenous IgGs in this system. In conclusion, we have established a versatile platform to reprogram human B cells to express engineered single-chain antibodies. It can be used with a variety of antigen-recognizing domains including VHH domains, scFvs and soluble receptor derivatives, expanding the range of antibodylike molecules that can be produced from the Ig locus. Multiplexed cassettes, comprising for example multiple VHH domains linked together by flexible linkers, can also be inserted and used to produce molecules with enhanced or multivalent functionalities. We believe the technology is broadly applicable and could be considered for any antibody-based therapy that would benefit from long-term *in vivo* production, including targets in cancer, autoimmune diseases, or other infectious diseases.

179. Immune Modulation Preceding AAV9GLB1 Gene Therapy Preserves the Possibility for Re-Dosing in Children with GM1 Gangliosidosis

Precill D'Souza[1], Kirsten E. Coleman[2], Caroline E. Rothermel[1], Manuela Corti[2], Cynthia J. Tifft[1], Barry J. Byrne[2]

[1]Office of the Clinical Director, NHGRI, National Institutes of Health, Bethesda, MD,[2]Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL

GM1 gangliosidosis is a rare, autosomal recessive, multisystem lysosomal storage disorder with relentless neurodegeneration and no effective therapy. AAV9 has broad distribution with intravenous administration and demonstrated tropism to the CNS. However, preexisting immunity to AAV9 decreases the effectiveness of transgene delivery and precludes the re-dosing of children as they grow. Here we present results of 6 Type II GM1 patients (ages 2.5 to 9 years) who received 1.5 x 10^{15} vg/kg AAV9-*GLB1* intravenously following a novel immune modulation pre-conditioning regimen to deplete CD19+ B cells and inactivate CD3+ T cells. All 6 patients received 4 intravenous doses of rituximab (375 mg/m^2) beginning 21 days prior to gene transfer and oral rapamycin (0.5-1 mg/m^2/day) to maintain a trough level of 5-10 ng/mL beginning 21 days prior to gene transfer and continuing 180 days following gene delivery. Methylprednisolone (1 mg/kg IV) was administered 60 minutes prior to gene transfer and prednisone (0.5mg/kg/d) was given orally for 3 days following dosing. CD19+ B cells were depleted following 2 does of rituximab in all 6 subjects with recovery beginning 3-6 months following gene delivery. There were no serious infections in any subject with the exception of one patient who developed bacterial sepsis from PICC line malfunction at 2 weeks following gene transfer. Quantitative immunoglobulins remained within normal ranges for all subjects through 6 months following gene transfer. Side effects of mucositis (4 patients) and mild neutropenia (3 patients) resolved without sequalae. IgM and IgG antibody titers to AAV9 did not change in 4 of 6 subjects. One subject, thought to be possibly pre-immune to AAV, had a delayed IgG response beginning at 14 days following vector administration that remained elevated through 180 days. A second subject had delayed IgM response to viral capsid that peaked at 30 days followed by a modest IgG response beginning at 30 days and persisted through 180 days. The pharmacokinetics of vector genome copies in serum for these 2 subjects were not different than the remaining 4 subjects who did not show a response. Following gene transfer, liver functions including alanine aminotransferase (ALT) and gamma-glutamyl-transferase (GGT) were within normal limits. Baseline aspartate aminotransferase (AST) was mildly elevated in all subjects consistent with our observations in a cohort of GM1 patients (mean=61.35 U/L, N=38). No subject had AST elevations greater than 3X their baseline value. C3 and C4 remained within the normal range and 3 subjects had mild thrombocytopenia (lowest value of 88 K/ mcL). Immune modulation was safe and well tolerated in our cohort and there were no complications in the days immediately following gene delivery. Two patients developed delayed antibody to the viral capsid, one of whom was likely pre-immune to AAV. Four patients showed no rise in antibody titer following gene transfer leaving open the possibility for re-dosing with the same AAV9 vector in the future.

New Gene Editing Technologies and Applications

180. Modulation of DNA Repair Pathways by HDR-CRISPR Promotes Seamless Genome Editing in Primary Human Hematopoietic Cells

Antonio Carusillo, Raul Schäfer, Manuel Rhiel, Daniel Türk, Tatjana I. Cornu, Toni Cathomen, Claudio Mussolino

Transfusion Medicine and Gene Therapy, Universitätsklinikum Freiburg, Freiburg

Im Breisgau, Germany

The CRISPR-Cas system is a robust platform for genome editing. The introduction of a DNA double-strand break (DSB) at precise gene locations can be exploited to achieve targeted gene knockout by harnessing the error-prone non-homologous end-joining (NHEJ) pathway. However, using CRISPR-Cas technology for precise genome editing via homology-directed repair (HDR) remains challenging, with HDR frequencies below the threshold required for clinical translation. Common strategies to increase HDR-mediated DSB repair include the use of chemicals either to inhibit NHEJ or to arrest the cells in those cell cycle phases when HDR is most active. However, the global effects of these drugs pose serious safety concerns if applied in clinically relevant settings. To address this issue, we devised a strategy to recruit HDR-promoting factors or NHEJ-inhibiting proteins to the DSB site. This is achieved via the direct fusion of particular protein-protein interaction domains to the Cas9 nuclease. We generated 16 different Cas9-fusion proteins (referred to as HDR-CRISPR) and extensively investigated their impact on DNA repair pathway choice by using two reporter systems, the traffic light reporter (TLR) and the BFPto-GFP (B2G) assay. These two assays allowed us to investigate the outcome of DNA repair mediated by a DNA donor supplied either as a plasmid or oligodeoxynucleotides (ODN) respectively. Our results indicate that HDR-CRISPRs enhanced HDR frequency 3-fold over baseline levels. The simultaneous reduction of NHEJ-mediated repair

resulted in a 5-fold increase in the HDR:NHEJ ratio when using our best performing HDR-CRISPR. Next, we assessed the capability of HDR-CRISPR to precisely integrate a large GFP expression cassette into the endogenous *AAVS1* safe harbor locus of K562 and Jurkat cell lines. Independently of the cell type, the use of HDR-CRISPR resulted in a 2.5-fold increase in targeted integration as compared to samples receiving the unmodified Cas9. The most efficient HDR-CRISPR fusion was then tested for its ability to promote HDR-mediated repair in clinically relevant primary human cells. HDR-CRISPR was delivered to T lymphocytes and hematopoietic stem cells (HSCs) in the form of RNA. Using an appropriate ODN as a repair template, we aimed at introducing a stop codon within exon 3 of the *CCR5* gene to generate immune cells resistant to HIV infection. HDR-CRISPR led to a 2-fold increase in precise genome editing events as compared to the use of an unmodified Cas9. In conclusion, our data support the hypothesis that DSB repair choice can be altered through the local recruitment of key DNA repair factors capable of either promoting HDR or inhibit NHEJ, also in clinically relevant cells.

181. Capturing and Characterizing Single Cell Allelic Heterogeneity of CRISPR-Cas9 Gene Editing *In Vivo*

Jesse A. Weber, Jonathan F. Lang, Alejandro M. Monteys, Beverly L. Davidson

Children's Hospital of Philadelphia, and University of Pennsylvania, Philadelphia,

PA

Translating CRISPR/Cas9 technology into *in vivo* therapeutic applications necessitates a strong understanding of the potential sites of delivery and the nature of subsequent gene editing events. Editingdependent reporter mouse strains (e.g. Ai6 and Ai14 (both containing lox-stop-lox-fluorescent protein at the *ROSA26* locus)) in combination with adeno-associated virus (AAV) delivered CRISPR/Cas9 offers a sensitive way to address some of these delivery and editing questions. In some occurrences, a deletion affecting both alleles of a gene may be desired. To assess the frequency and fidelity of biallelic editing, we generated compound Ai14/Ai6 heterozygote mice. Injection of AAVs encoding Cas9 protein and gRNAs targeted to the transgenic *ROSA26* locus will edit the stop cassette and activate tdTomato and/or ZsGreen from the Ai14 and Ai6 reporter alleles, respectively. Interestingly, after AAVgRNA delivery, the most frequent outcome was that only one of the two reporter alleles was activated in transduced hepatocytes, suggesting allelic heterogeneity within single cells following *in vivo* editing. The experiment was repeated *in vitro* using Ai14/Ai6 mouse embryonic fibroblasts and SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Following transfection, we again observed heterogeneity in reporter activation. Next, we used flow to separate cells into double positive (tdTomato+/ZsGreen+), single positive (tdTomato+/ZsGreen- or tdTomato-/ZsGreen+), or double negative (tdTomato-/ZsGreen) populations and sequenced DNA from tdTomato-/ZsGreen+ single positive cells at the Ai14 (non-expressing) and Ai6 (expressing) alleles. As expected, the Ai6 allele contained the desired CRISPR-mediated deletion of the stop cassette. However, at the non-expressing Ai14 allele, the most common editing outcome was indels at both gRNA cuts sites without deletion of the stop cassette. Contrary to our expectation, the apparent reason for non-activation at the Ai14 allele was not lack of editing, but rather, undesired editing. The NHEJ DNA-repair inhibitor,

Ku57788, increases CRISPR deletion efficiency *in vitro*. To test its effects on biallelic deletions, Ai14 /Ai6 mouse embryonic fibroblasts were treated with Ku57788 and transfected with SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Flow-sorted cells exhibited a marked increase in single positive cells of both reporter alleles, but most importantly exhibited a near 2-fold increase in the number of cells with a successful biallelic deletion. This suggests that efforts to transiently inhibit DNA repair may increase biallelic CRISPR deletion efficiency.

182. Dual-HDR Editing Strategies for the Development of Islet-Specific Regulatory T Cells (EngTregs) for Restoration of Immune Tolerance in Type 1 Diabetes

Ahmad Boukhris[1], Peter Cook[1], Soo Jung Yang[2], Iram Khan[1], Jane Buckner[2], David Rawlings[1]

[1]CIIT, Seattle Children's Research Institute, Seattle, WA,[2]Benaroya Research Institute, Seattle, WA

Adoptive transfer of engineered regulatory T cells (EngTregs) represents an emerging therapeutic approach to promote immune tolerance in the setting of stem cell or solid organ transplantation,

and in autoimmune diseases including type 1 diabetes (T1D). We previously developed robust methods to generate polyclonal EngTregs from CD4+ T cells by homology-directed-repair (HDR)-based gene editing. This approach introduces a ubiquitous promoter (MND) into the *FOXP3* locus downstream of the Treg-specific demethylated region (TSDR), resulting in high level FOXP3 expression and stable Treg-like phenotype and function. Polyclonal EngTregs cells generated by this approach are beneficial for systemic autoimmune conditions (including IPEX and GvHD) but lack the fine-specificity required to control tissuespecific autoimmune diseases driven by locally-expressed self-antigens. In this study, we describe a robust dual-HDR approach to generate and enrich antigen-specific EngTregs cells. Knock-out of the endogenous T cell receptor alpha constant (*TRAC*) gene and replacement with an islet-specific TCR is predicted to generate EngTregs capable of localizing to APCs expressing islet antigens in the pancreatic lymph nodes and/or pancreas. We evaluated three strategies for introducing an islet-specific TCR into the *TRAC* locus in parallel with HDR-editing of the *FOXP3* locus. To overcome the anticipated lower efficiencies of dual-HDR editing, we generated HDR donor cassettes designed to simultaneously introduce a heterodimeric, chemically-induced signaling complex (CISC) that mimics IL-2 signaling in response to an exogenous dimerizer, rapamycin. For our dual-HDR approach, the dimerizing components of the CISC cassette were introduced via two separate locus-specific editing events (in *TRAC* and *FOXP3*), thereby enabling specific selection and enrichment of only the dual-edited, islet-specific EngTregs. We first attempted a *TRAC* knock-in cassette incorporating an MND promoter, the full islet-TCR coding domain, and half of the CISC cassette. This strategy produced dual-edited cells that enriched in response to CISC dimerization. However, due to the large size of the TRAC-targeting repair template, the initial editing rates were low. To reduce the size of the HDR cassette, we pursued a promoter capture strategy, introducing the islet TCR downstream of the endogenous TRAC promoter. Notably, split-CISC test constructs using fluorophore markers showed that the these dual-edited cells failed to enrich, implying that CISC functionality requires high surface density of the synthetic receptor. Taking these findings into account, we designed an in-frame knock-in construct with an MND promoter driving CISC expression followed by the partial islet-TCR sequence (bearing the TCR beta chain and the alpha chain variable region) hijacking the endogenous alpha constant gene. Using this approach, we observed strong CISC-based enrichment of dual positive, antigen specific, EngTreg cells. After culturing cells in dimerizer, edited cells enriched from initial dual HDR-editing rates of 5-10% to >80% within 7 days, and these populations expressed high-levels of FOXP3 and relevant Treg surface markers. Our findings demonstrate that dualHDR editing using a 'split IL-2 CISC' is a viable strategy for generating antigen-specific EngTreg cells. This approach has the capacity to deliver targeted immune tolerizing therapy to treat or prevent T1D and is likely to be easily adapted for use with alternative TCRs (or CARs) designed to target tissue-specific autoimmune or inflammatory diseases.

183. A Dimeric, Luminescent Biosensor for Imaging Unique DNA Sequences in Individual Cells

Nicholas G. Heath[1], David J. Segal[1], Henriette O'Geen[1], Nicole B. Coggins[1], Jacob Corn[2]

[1]Genome Center, UC Davis, Davis, CA,[2]Institute of Molecular Health Sciences, ETH, Zurich, Switzerland

One of the most prominent bottlenecks in the gene editing process is the ability to identify and isolate individual cells with desired edits within a population of treated cells. Current approaches typically require time-consuming and labor-intensive single cell isolation and population expansion followed by destruction of some portion of an expanded cell population for downstream *in vitro* analysis of DNA sequence content. Cell types that exhibit low efficiencies in transfection, editing, single cell isolation, or population expansion can be particularly challenging. To compound this problem, homology directed repair (HDR) can exhibit extremely low efficiency in certain cell types. A promising alternative to these and other destructive DNA detection assays could be the direct biosensing of edited DNA sequences in living cells. In recent years, an extensive arsenal of biosensing tools has been developed based on the clustered regularly interspaced short palindromic repeat (CRISPR) platform, including those that detect the presence of specific DNA sequences both *in vitro* and in live cells. In fact, the CRISPR/Cas gene editing system has been extensively modified for imaging endogenous genomic loci, but the vast majority of current approaches utilize monomeric fluorescent reporter-based biosensors, such as dCas9-GFP. A major drawback to these systems is that each monomeric sensor produces a signal whether bound to its target DNA or not, resulting in a high fluorescent background that negatively impacts the signal-to-noise ratio. For this reason, such "always-on" sensors must rely on obtaining a high local concentration of probes to distinguish signal from noise, limiting their use to highly repetitive elements that can be targeted by one gRNA or to unique sequences targeted by 50 or more gRNAs. In contrast, dimeric "turn-on" DNA biosensors offer the possibility of achieving signal production solely upon binding of two subunits to the target DNA and reassembly of a bright reporter. Furthermore, luminescent reporters are an attractive alternative to fluorescent reporters in biosensing experiments as cellular background luminescent signal is essentially nonexistent. This is due to the different nature of light production in luminescent reporters where a catalytic reaction of an enzyme with its substrate produces light, eliminating the need for exogenous excitation light.Taking these points into consideration, we have developed a luminescence-based, dimeric DNA sequence biosensor that provides a sensitive readout for DNA sequences through proximity-mediated reassembly of two independently optimized fragments of NanoLuc luciferase (NLuc), a small, bright reporter. Reconstitution of NLuc becomes more favorable upon binding of two guide RNAs (gRNAs) to two DNA target sites with a defined orientation and spacing. Using this "turn-on" probe, we demonstrate rapid and sensitive detection of as low as 190 amol transfected target DNA, presenting a reliable approach for DNA biosensing. Across several cell-based delivery approaches, we were able to achieve approximately 2.5 - 20-fold increase in signal in live populations of cells transfected with the dimeric biosensor and various target DNA scaffolds compared to populations transfected with the dimeric biosensor but no target DNA. The future goals for this system include detection of single-copy genomic sequences at endogenous loci and validation of gene sequence changes after genome editing experiments in live cells.

184. Efficient CRISPR-Cas9-Mediated Gene Knockout and Interallelic Gene Conversion in Human Induced Pluripotent Stem Cells Using Non-Integrative Bacteriophage-Chimeric Retrovirus-Like Particles

John De Vos[1], Joffrey Mianné[1], Chloé Nguyen Van[1], Chloé Bourguignon[1], Mathieu Fieldès[1], Engi Ahmed[1], Christine Duthoit[2], Nicolas Martin[3], Alexandra Iché[3], Régis Gayon[3], Florine Samain[3], Lucile Lamouroux[3], Pascale Bouillé[3], Arnaud Bourdin[1], Said Assou[1]

[1]U1183, INSERM, Montpellier, France,[2]U1183, Flash Therapeutics, Toulouse, France,[3]Flash Therapeutics, Toulouse, France

The combination of CRISPR/Cas9 technology with human induced pluripotent stem cells (hiPSC) has tremendous potential for basic research and cell-based gene therapy. However, fulfilling these promises relies on our capacity to efficiently deliver exogenous nucleic acids into these cells and harness the repair mechanisms induced by the nuclease activity.Since gene editing systems require low and shortterm expression in order to avoid off-target effects, RNA delivery is favored over DNA delivery. RNA delivery presents major advantages compared to DNA delivery, being that it is completely safe and devoid of any recombination events in the host genome. It is actually the most versatile, flexible, and safe mean for human therapy.Here, we investigated the potential of bacteriophage-chimeric retrovirus-like particles for the non-integrative delivery of RNA molecules in hiPSC. We found that these particles efficiently convey RNA molecules for transient expression in hiPSC, with minimal toxicity and without affecting cell pluripotency and subsequent differentiation. We then used this system to transiently deliver the CRISPR-Cas9 components (Cas9 mRNA and sgRNA) to generate a gene knockout with a high indel level (up to 85%) at several loci into hiPSC. Strikingly, when using an allele-specific sgRNA at a locus harboring compound heterozygous mutations, the targeted allele was not altered by NHEJ/MMEJ, but was repaired at high frequency using the homologous wild type allele, suggesting interallelic gene conversion.Our results highlight

the potential of bacteriophage-chimeric retrovirus-like particles to efficiently deliver RNA molecules in hiPSC, and describe for the first time genome engineering by gene conversion in hiPSC. Harnessing this DNA repair mechanism could facilitate the therapeutic correction of human genetic disorders in hiPSC.

185. Triggering P53 Activation and Trapping of Transcriptionally Active Recombinant AAV Sequences Are Inadvertent Consequences of HSC Genome Editing

Samuele Ferrari*[1], Aurelien Jacob*[1,2], Marianne Laugel[3], Véronique Blouin[3], Stefano Beretta[1], Valentina Vavassori[1], Luisa Albano[1], Ivan Merelli[4], Eduard Ayuso[5], Oumeya Adjali[3], Magalie Penaud-Budloo[3], Pietro Genovese[1], Luigi Naldini[1,6]

[1]San Raffaele Telethon Institute for Gene Therapy, Ospedale San Raffaele, Milan, Italy,[2]University of Milano-Bicocca, Monza, Italy,[3]INSERM UMR 1089, University of Nantes, Nantes, France,[4]National Research Counsil, Segrate (MI), Italy,[5]INSERM UMR 1089, University of Nantes, Milan, France,[6]Vita-Salute San Raffaele University, Milan, Italy

Gene editing by homology-directed repair (HDR) in human hematopoietic stem cells (HSC) holds therapeutic potential for the treatment of hematological diseases by *in situ* correction of pathogenic mutations or site-specific integration of a transgene expression cassette. Induction of DNA double strand breaks by nucleases (e.g. CRISPR/
Cas ribonucleoprotein) and cell transduction with single-stranded Adeno-Associated Vector serotype 6 (ssAAV6) to deliver the donor template for HDR cumulativelytrigger a robust p53-dependent DNA damage response (DDR), which delays HSC proliferation, lower the yield of edited cells and ultimately results in oligoclonal reconstitution after xenotransplantation. Transient p53 inhibition released cell cycle block and improved size and clonal composition of the human graft. Although ssAAV6 is the major contributor to p53 pathway activation, the elements responsible for such DDR signaling as well as their persistency in edited HSC have not been fully characterized. We did not observe significant differences in kinetics or extent of p53 pathway activation due to targeted loci, transgene cassette or ssAAV6 purification process, suggesting a shared viral component as DDR trigger. However, HSC edited and transduced with genomefree ("empty") AAV6 did not promote p53 response over nucleaseonly treated cells and robustly engrafted in immunodeficient mice, thus ruling out capsid-dependent effect and confirming that ssAAV genomes can activate the p53 pathway. Delivery of the HDR template by self-complementary (sc)AAV6 triggered a more prolonged p53 transcriptional response and further reduced repopulation capacity of edited cells compared to ssAAV6-edited ones. Since recombinant (r) AAV genomic elements may trigger detrimental cellular responses in cultured HSC, we evaluated persistence of vector sequences in edited cells by deep sequencing the edited allele. A low fraction of HSCs unexpectedly carried on-target trapping of rAAV DNA fragments despite the transient delivery of editing components in these actively proliferating target cells. This finding was consistent across different editing loci and transgene cassettes and was confirmed in other human primary hematopoietic cells. Edited HSC with on-target trapping of rAAV DNA fragments engrafted in immunodeficient mice and

persisted long-term after transplant (median allele frequency < 0.5%). Sequence alignment of trapped fragments to the parental rAAV genome showed prevalence of the inverted terminal repeats (ITR) including the Rep binding element and the terminal resolution site. Of note, hematopoietic cells transduced with ssAAV6 harboring a promoterless reporter cassette showed low but detectable ITR-dependent transcriptional activity. We are currently investigating the underlying mechanism of such rAAV fragment trapping and whether

ITR-driven transcription may contribute to the adverse impact of AAV on HSC. This work will help to design strategies in order to overcome these inadvertent consequences of HDR template delivery and improve the efficiency and predicted safety of gene editing.

186. Accurate Quantification of CRISPR/Cas9 Induced Large Deletions, Insertions and Chromosomal Rearrangements Using SMRT Sequencing with Unique Molecular Identifiers

So Hyun Park, Mingming Cao, Gang Bao
Bioengineering, Rice University, Houston, TX

CRISPR/Cas9 induced double-stranded breaks (DSBs) can result in complex gene editing outcomes, including small insertions and deletions (INDELs), large INDELs, and complex chromosomal rearrangements, which may have pathogenic consequences. The current gold standard for quantifying Cas9 activity is short-range PCR amplification followed by next-generation sequencing (NGS). This method relies on amplifying short sequences around the cut site, thus significantly limits the accuracy in quantifying alleles with large INDELs or chromosomal rearrangement or loss. We previously developed the LongAmp-Seq (Long-range PCR Amplification based Sequencing) assay, a combination of long-range PCR and NGS. LongAmp-seq utilizes the widespread availability of Illumina NGS platform and provides high throughput discovery of large deletions as well as small INDELs at the Cas9 cut-site. However, we found that small amplicons carrying large deletion events were falsely overrepresented during long-range PCR, making accurately quantifying large deletions difficult. Here, we used *S. pyogenes* (Spy) CRISPR gRNA/Cas9 (RNP) to target beta-globin gene (*HBB*) in hematopoietic stem and progenitor cells (HSPCs) from patients with sickle cell disease, adapted the dualunique molecular identifiers (UMI) tagging for long-read amplicon sequencing, and analyzed the induced mutagenesis. The 5.5 kb *HBB* region around the Cas9 cut-site was dual-UMI tagged using two PCR cycles, followed by a second and third PCR to enrich the dual UMI-tagged template molecules. The SMRT-bell library composed of RNP-treated and untreated samples was sequenced on a PacBio Sequel II 8M flowcell in circular consensus sequencing (CCS) mode. The PacBio subreads were converted to HiFi reads and subjected to UMI-based de-duplication and variant calling. The raw PacBio CCS reads without UMI clustering generated 300k reads with full-length coverage and dual UMIs of the correct length and pattern. Reads were binned based on the concatenated UMI pair. In RNP treated sample, we detected diverse large deletions of up to 4 kb and insertions up to 600 bp, and complex local rearrangement events. An overrepresentation of large deletion fragments was demonstrated by higher UMI coverage. After UMI-based de-duplication, the frequency of deletions larger than 100bp decreased from 40% to 31%, and the frequency of deletions larger than 1kb decreased from 15% to 11%. We quantified a diverse array of HBB gene editing outcomes including large deletions/insertions and complex rearrangements using the dual-UMI long-read amplicon sequencing. Our results showed that the current assessments of gene editing rates using short-range PCR based NGS could miss a substantial proportion of Cas9-induced mutations, leading to a large error in gene editing frequencies. With the rapid development of CRISPR-based therapeutic applications, there is an unmet need to develop a simple, accurate and reliable method to determine the complex gene editing outcomes and study the potentially detrimental consequences of unexpected mutations.

Novel AAV Biology and Platform Technologies

187. Effect of pH and Temperature on AAV2 Capsid Structure and Stability

Joshua Alexander Hull[1], Justin Kurian[1], Antonette Bennett[1], Balasubramanian Venkatakrishnan[1], Mario Mietzsch[1], Paul Chipman[1], Chen Xu[2], Duncan Sousa[3], Mandy Janssen[4], Timothy Baker[4], Robert McKenna[1], Mavis Agbandje-McKenna[1]
[1]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,[2]Medical School Cryo-EM Core Facility, University of Massachusetts, Worcester, MA,[3]Department of Biological Sciences, Florida State University, Tallahassee, FL,[4]Department of Chemistry and Biochemistry and Division of Biological Sciences, University of California San Diego, San Diego, CA

Adeno-associated viruses (AAV) and recombinant AAVs (rAAVs) must enter the host cell by endo-lysosomal trafficking, where the capsid is exposed to an increasingly acidic (pH 7.4-5.5) environment[1]. During this process, a viral phospholipase A_2 (PLA_2) enzyme, within the capsid viral protein 1 (VP1), functions to allow the capsid to escape the endo-lysosomal pathway to traffic to the nucleus for uncoating and genome replication[1,2]. For this function, the PLA_2 undergoes a switch from the capsid interior to exterior, necessitating proposed capsid rearrangements which are currently poorly understood[3,5,6]. This process can be induced with heat (~60 ºC)[3,5,6] as a surrogate. Towards understanding the necessary capsid rearrangements associated with PLA_2 externalization, we present the structure of AAV2 virus-like particles determined under 10 different conditions to 2.7-4.0 Å resolution, including pre-incubation at varying temperatures (4, RT, 55, and 60 ºC) and pH (7.4, 6.0, 5.5, and 4.0), by single particle reconstruction cryo-electron microscopy. At RT pre-vitrification, capsid rearrangements are observed at the 2-fold axes in previously described variable regions (VR) III and IX at pH 5.5 and 4.0. In addition, unmodeled density, previously interpreted as the unique region of VP1 (VP1u) containing the PLA_2[4,5], was observed at room temperature irrespective of pH. At neutral pH with 60 ºC previtrification, the rearrangement of the 2-fold VR IX can be partially reproduced. We demonstrate that low pH conditions, and heat as a surrogate, create capsid dynamics at the 2-fold axis suggesting this region as well as the 5-fold pore are essential for PLA_2 externalization and function. [1]Florian Sonntag *et al.* 2006 [2]Anne Girod *et al.* 2002 [3]Svenja Bleker *et al.* 2005 [4]Stephanie Kronenberg *et al.* 2001 [5]Stephanie

[1] Gene Transfer Technologies, REGENXBIO, Rockville, MD,[2]Target Discovery, REGENXBIO, Rockville, MD

188. Development of a Split Rep/Cap System to Improve AAV Capsid Production

Derek Carbaugh[1], Audry Fernandez[1], Lester Suarez[1], Richard J. Samulski[1,2]

[1]R&D, AskBio, Durham, NC,[2]Gene Therapy Center, University of North Carolina,

Chapel Hill, NC

Adeno-associated virus (AAV)-based gene therapy is rapidly becoming established as a clinical therapy for genetic diseases. Despite their great promise, one of the largest limitations to their widespread use is the inability to manufacture enough high quality AAV vectors quickly and cost effectively. The wild-type AAV genome comprises inverted terminal repeats that flank two open reading frames (ORFs): *rep* and *cap*. The *rep* ORF includes four overlapping genes encoding the proteins required for AAV replication and packaging, whereas the *cap* ORF contains overlapping sequences encoding viral proteins 1, 2, and 3 (VP1-VP3), which interact to form a capsid. Recombinant AAV (rAAV) is constructed by replacing the *rep* and *cap* genes with a therapeutic gene; consequently, an adequate source of *rep* and *cap* proteins must be provided *in trans* for the rAAV lifecycle to proceed. Under normal conditions, large Rep78 and small Rep52 are derived from two distinct promoters, p5 and p19, respectively. The transcribed mRNAs then undergo splicing to form Rep68 and Rep40. WT AAV expresses VP1-3 from its p40 promoter, which overlaps with the *rep* ORF. These VPs, which only differ in their N-terminus, are produced by alternative splicing and leaky scanning from one reading frame to achieve a molar ratio of VP1:VP2:VP3 = 1:1:10. However, inefficient splicing may result in inefficient *cap* production and the best VP ratio for the most efficient assembly of correct viral capsids is still uncertain. To overcome the problem of inefficient splicing and attempt to optimize VP ratios we have split the *rep* and *cap* ORFs by cloning *cap* upstream of *rep*, driven by a pCMV promoter. Additionally, since Rep78 and Rep52 have been shown to be sufficient for replication, we modified *rep* to produce a codon optimized *rep* (coRep), ablating donor and acceptor sites for Rep68 and Rep40 splicing, as to only produce Rep78 and Rep52. Our split coRep/Cap system will allow us to optimize the expression level of both the *rep* and the *cap* proteins through codon optimization, modification of Kozak sequences, promoters, transcription start sites, post-translational modifications, etc, while avoiding altering protein splicing, to improve production of AAV capsids for use in gene therapy.

189. Intravenous Administration of a Barcoded and Pooled AAV Library for the Comprehensive Characterization and Comparison of Capsid Tropisms

April R. Giles[1], Samantha A. Yost[1], Elad Firnberg[1], Jenny M. Egley[1], Karolina J. Janczura[2], William M. Henry[2], Ye Liu[1], Subha Karumuthil-Melethil[2], Andrew C. Mercer[1]

As the breadth and quantity of potential applications for adenoassociated viral (AAV) vector-mediated gene therapy expands, so does the demand for capsids with unique properties tailored to address each indication. REGENXBIO's NAV® Technology Platform is comprised of over 100 proprietary AAV vectors. We sought to thoroughly evaluate the potential of these AAVs to perform efficiently as gene therapy vectors. The productivity of each vector was evaluated by triple plasmid transfection into suspension HEK293 cells, screening primarily for supernatant yield and secondarily on cell pellet yield. Based on these criteria, 56 NAV® Platform vectors were selected for inclusion in the study; an additional 49 engineered AAV variants and 13 commonly used AAVs were also included. Each AAV was produced individually, packaging a CAG-GFP transgene cassette with a unique barcode, and pooled in approximately equal concentrations to generate a library of 118 barcoded AAVs. This library was administered intravenously to both mice (C57BL/6) and non-human primates (NHPs, cynomolgus macaques) at a dose of 1.77e13 GC/kg. Next-generation sequencing (NGS) was used to determine the relative abundance of barcoded genomes and transcripts present in a number of target tissues, allowing for comprehensive characterization of each capsid in the pool, direct comparison between capsids of interest, evaluation of clade-based transduction patterns, and translation of findings between species. Three, six, and twenty-four hours after dosing, we sampled NHP blood to determine the rate of vector clearance. AAV6 was the most rapidly cleared vector, with no genome copies detected at any time point post administration. Clade E vectors (e.g. AAV8, rh.10) trended towards quicker blood clearance compared to members of other clades, while AAV variants designed to de-target the liver, as well as AAV4, remained in circulation longer. In the livers of both species, vectors belonging to clade E were the most enriched relative to vectors from other clades. Clade C vectors were the least enriched in the livers of both species. Clade A members transduced mouse liver poorly but efficiently transduced NHP liver, largely driven by AAV6. We identified a previously uncharacterized clade E AAV that was as efficient as AAV8 in transducing both mouse and NHP liver but transduces the peripheral organs from both species 8-38-fold less efficiently than AAV8; this AAV was significantly less tropic for the central and peripheral nervous systems than AAV8 in both species. Heart transduction was dominated by clade F vectors (e.g. AAV9) in both species. Clade E vectors were enriched in mouse heart, while clade A, B, and C vectors transduced this tissue poorly, but these trends did not translate to NHP heart. Additionally, we identified a clade C AAV that transduced NHP heart with similar efficiency to AAV9 but, unlike AAV9, failed to efficiently transduce the liver (over 20-fold reduction) and other peripheral tissues; however, this vector did not efficiently transduce mouse heart. We also discovered that a single fringe AAV (not assigned to an existing clade) accounted for over 35% of all AAV genomes and transcripts in the mouse lung, while this property did not translate to the NHP lung. Taken together, these findings highlight the importance of direct, simultaneous comparison of the performance of these AAVs to several commonly used AAV vectors in multiple species and across tissues to fully characterize a given variant. From this study, we have identified

capsids uniquely suited to address specific indications while also identifying trends to steer future capsid engineering efforts.

Novel AAV Biology and Platform Technologies

190. AAV Capsid Property Estimation Is Improved by Combining Single-Molecule ID Tags and Hierarchical Bayesian Modeling of Experimental Processes

Kathy Lin, Jakub Otwinowski, Brian Tobin, Sam Wolock, Jamie Kwasnieski, Cem Sengel, Roza Ogurlu, Eryney Marrogi, Megan Cramer, Sylvain Lapan, Anna Wec, Chris Reardon, Nishith Nagabhushana, Helene Kuchwara, Heikki Turunen, Shireen Abestesh, Ilke Akartuna, Alexander Brown, Farhan Damani, Jorma Gorns, Jeff Jones, Elina Locane, Stephen Malina, Hanna
Mendes Levitin, Patrick McDonel, Stephen Northup, James Oswald, Amir Shanehsazzadeh, Sam Sinai, Michael Stiffler, Flaviu Vadan, Adrian Veres, Lauren Wheelock, Justin Yan, Eric Kelsic, Jeff Gerold
Dyno Therapeutics, Cambridge, MA

AAV capsid engineering has been accelerated by the application of high-throughput synthesis and sequencing technologies to measure the properties of many barcoded capsid variants in parallel. While these techniques significantly increase the number of capsid variants that can be screened, they also introduce errors that decouple barcodes from designed capsids and obscure the measurement signal. These errors include cross-packaging, template switching, and errors in DNA synthesis. To address these issues, we developed a paired library construction and Bayesian modeling strategy that identifies sets of sequences which are likely to contain errors. Incorporation of additional random DNA IDs subdivides the barcoded sequence populations. Each barcode-ID can then be tracked through the stages of plasmid production, capsid assembly, and transduction. We probabilistically model the measurement process and sources of decoupling between barcode-IDs and designed capsids and infer parameters describing the different production, transduction, and error rates, with Bayesian methods. In particular, we leverage probabilistic programming and stochastic variational inference to infer complex models of these mechanisms on large data. To combine information from multiple experiments in a principled manner, we model variation between samples and jointly model production and transduction rates. Notably, we infer for each barcode-ID whether it is correctly linked with its designed capsid sequence, which we verify experimentally with independent measurements. Thanks to this identification of errors, inferred production and transduction rates are significantly less noisy, as shown by the distribution of wildtype and stop codon controls. For an in vitro transduction assay, our analysis reduced the false positive transduction rate among VP1 stops and narrowed the set of high-performing variants without impacting wild-type controls. We verify the accuracy of our uncertainty estimates with replicate data and low-throughput validation data. ID tracking also enables estimation of new quantities of interest, such as the numbers of transfected and transduced cells per capsid variant. In addition, accurate uncertainty quantification is critical for identifying promising variants for further study and can be utilized for improving models that predict capsid properties from their sequence. We explore the utility of uncertainty estimates by training a simple sequence-function model jointly with our experimental process model.

The resulting model learns the effect of mutations on capsid properties by integrating information from multiple variants, which may be necessary when measurements are noisy. Overall, our approach can be simply incorporated into many barcode-based assays of capsid function and significantly improves estimation of packaging and transduction in large, barcoded AAV libraries.

191. Hydroxylation of *N*-acetylneuraminic Acid Influences the *In Vivo* Tropism of *N*-linked Sialic Acid-Binding Adeno-Associated Viruses AAV1, AAV5 and AAV6

Estrella Lopez-Gordo, Alejandro Orlowski, Arthur Wang, Thomas Weber
Cardiovascular Research Center (CVRC), Icahn School of Medicine at Mount Sinai, New York, NY

Adeno-associated virus (AAV) vectors are promising candidates for gene therapy. However, a number of recent preclinical large animal studies failed to translate into the clinic. This illustrates the formidable challenge of choosing the animal models that promise the best chance of a successful translation into the clinic. Several of the most common AAV serotypes use sialic acid (SIA) as their primary receptor. However, in contrast to most mammals, humans lack the enzyme CMAH, which hydroxylates Neu5Ac into Neu5Gc. Here, we investigate the tropism of SIA-binding AAV1, 5, and 6 in wild-type (WT) and CMAH knock-out ($CMAH^{-/-}$) mice. All three serotypes showed significant differences in tropism in $CMAH^{-/-}$ when compared to WT mice. Transduction of heart was 5.8-fold, 3-fold and 5.4-fold lower in $CMAH^{-/-}$ mice for AAV1, 5 and 6, respectively. Importantly, the most dramatic difference in transduction was observed for AAV5 in the skeletal muscle, where transduction was 30-fold higher ($p<0.001$) in $CMAH^{-/-}$ mice. Expression in the brain, kidney and lung was overall much lower than that observed in heart, liver and skeletal muscle for all the AAV serotypes tested, and only AAV1 showed 1.4-fold lower transduction of $CMAH^{-/-}$ mice lung. The staining pattern and abundance of α2-3/6 *N*-linked SIA-containing glycoproteins that serve as AAV1, 5 and 6 receptors showed no dramatic differences in tissue between $CMAH^{-/-}$ and WT mice. Together, these data suggest that tropism of AAV1, 5 and 6 may not only depend on the type of linkage (e.g: α2-3 or α2-6) between the glycans that form *N*-linked SIA, as described in the literature, but it also may be influenced by particular modifications on these glycans such as the hydroxyl group that is present on Neu5Gc but missing on Neu5Ac. Thus, the relative abundance of Neu5Ac and Neu5Gc in tissue may play a role in AAV tropism. These findings are important for a deeper understanding of the mechanisms dictating AAV tropism and are fundamental for the

identification of translationally relevant animal models for AAV research.

192. High-Throughput Screening of AAV Productivity to Enable Rapid Capsid Characterization

Jenny M. Egley, April R. Giles, Samantha A. Yost, Elad
Firnberg, Chunping Qiao, Kirk Elliott, Randolph Qian, Devin S. McDougald, Ye Liu, Olivier Danos, Andrew Mercer
REGENXBIO Inc., Rockville, MD

Adeno-associated virus (AAV) mediated gene therapy is a rapidly growing field with immense potential to deliver curative treatments to patients with unmet needs. Hundreds of unique, naturally occurring AAV capsid sequences, as well as innumerable engineered capsids, have been identified; yet, outside of the commonly used capsid serotypes (AAV1-12), there is a general lack of characterization of these capsids for overall production, packaging efficiency, and other properties impacting their implementation. One challenge for screening large numbers of AAV capsids is the low throughput of triple plasmid transfection production systems. Here we describe a high-throughput, 96-well platform that allows us to screen hundreds of capsids simultaneously to define potential capsids of interest more rapidly. This method produces AAV vectors by triple plasmid transfection of a suspension HEK293-based system in 1 mL culture volume using 96-well deep well plates sealed with gas-permeable membranes. The plates are incubated in an INFORS Multitron shaking incubator modified to a 3 mm orbital throw at 1000 rpm to achieve the optimal oxygen and CO_2 levels. Initially, we screened approximately 150 naturally occurring capsids packaging CAG-firefly luciferase (total genome length of 4.0 kb) in duplicate and found minimal well-to-well or plate-to-plate variation, demonstrating this system's robustness. Post-production harvest and in-plate cell lysis allowed the evaluation of total and secreted AAV titer concurrently. The overall productivity in the supernatant and lysed cell pellet of these vectors was comparable to that of those produced in cultures of larger volume grown in a shaker flask under the same triple transfection conditions after determining viral genome titer by quantitative polymerase chain reaction (qPCR), further validating this method. Next, we screened the same set of capsids for their ability to package a larger transgene, with a total genome length of 4.7 kb, to determine the impact of genome length on vector yield. We found several highly homologous capsids within the same clade demonstrated varying levels of secretion and overall production, suggesting the size of the transgene cassette has a significant impact on the yield of vector in a capsid-specific fashion. This screening platform can be further employed to identify residues critical for AAV structure-function relationships, such as secretion, via residue swapping or alanine scanning experiments when such approaches would not be reasonably feasible in a lower throughput method. Under these described conditions, several hundred AAV vectors can be screened rapidly in parallel with reduced hands-on time and potential for automation, demonstrating that this system is a valuable tool for accelerating capsid engineering efforts to develop the next-generation AAV vectors for gene therapy.

193. AAV-GPseq Analysis of Vectors from HEK293 and BEV/Sf9 Production Platforms Reveals Differential Genome Heterogeneity and Enrichment of Potential Innate Immune DNA Epitopes in Empty Capsids

Ngoc Tam Tran[1,2], Kristina Weber[3], Suk Namkung[1,2], Eduard Ayuso[4], Oumeya Adjali[4], Cécile Robin[4], Eric Devine[4], Veronique Blouin[4], Magalie Penaud-Budloo*[4],
Guangping Gao*[1,2], Phillip W. L. Tai*[1,2]
[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]Pacific Biosciences
Inc., Menlo Park, CA,[4]INSERM UMR 1089, University of Nantes, Nantes, France

The use of adeno-associated virus (AAV) vectors in gene therapy has shown promise for treating a wide-range of diseases, especially for rare diseases. As a consequence, AAV vector production has been pushed to meet the high standards needed to deliver safe and effective doses to patients. In the past two decades, vector manufacturing has made remarkable advancements to meet large-scale production demands for clinical trials and approved treatments in today's markets. AAV vectors have been extensively studied to assess their safety and efficacy. The presence of empty AAV capsids and particles containing "inaccurate" vector genomes in preparations have been a subject of concern for nearly 20 years. Whether they are considered as contaminants or can act as immune decoys to improve therapy depends on the criteria for assessing vector potency and efficacy. Nonetheless, the current trend for AAV vector manufacturing is to obtain the highest purity of full particles as possible. Several methods are employed to separate empty capsids from full particles; but thus far, no single technique can guarantee empty or intermediate (genome truncation) capsidfree preparations. Therefore, these non-full particles remain critical concerns. Unfortunately, the exact vector genome compositions of full, intermediate, and empty capsids, remain largely unknown. In this work, we use AAV-Genome Population Sequencing (AAVGPseq) to explore the compositions of DNase-resistant, encapsidated vector genomes produced by two common production pipelines: triple-transfection in human embryonic kidney cells (HEK293) and baculovirus expression vectors in *Spodoptera frugiperda* insect cells (BEV/Sf9). As we have found previously, "full particles" can contain truncated genomes irrespective of the production and purification methods that generate them; and as expected, intermediate capsids are predominately composed of inaccurate or chimeric genomes. We also demonstrate that empty particles purified by CsCl gradient ultracentrifugation are not truly empty, but are instead packaged with genomes comprised of a single truncated and/or unresolved inverted terminal repeat (ITR). Our findings suggest that the frequency of these "mutated" ITRs correlate with the abundance of inaccurate genomes, which may yield a higher degree of empty capsids. For example, trident-shaped mutant ITRs are carried by unresolved species and form self-complementary AAV genomes. Intriguingly, our results show that vectors manufactured by HEK293 production